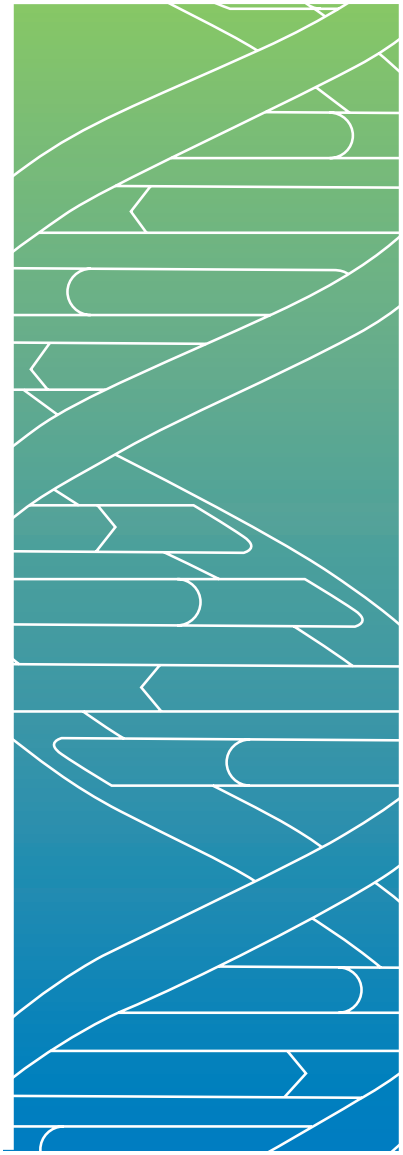
AMGEN® Biotech Experience

Scientific Discovery for the Classroom

FOUNDATIONS OF BIOTECH



ABRIDGED GENETIC ENGINEERING SEQUENCE

Student Guide

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AMGEN® Foundation

TABLE OF CONTENTS

ABOUT THE AMGEN BIOTECH EXPERIENCE

Genetic engineering is a branch of biotechnology that uses special procedures and techniques to change an organism's DNA. This ability has had a huge impact on the field of medicine, as genetically modified bacteria can make human insulin (the hormone responsible for regulating glucose levels in the blood) and other life-saving products. It's rare for high school students to have the chance to learn about and actually practice the procedures and techniques that are the foundation of the biotechnology industry—but in this program, you will have just that opportunity. As you work in the laboratory and carry out the very experiments that led to breakthroughs in biotechnology, you will gain hands-on experience with producing genetically modified bacteria.

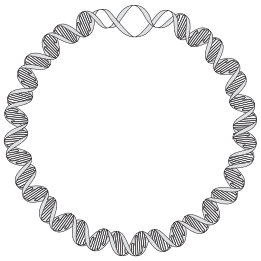
The procedures in this program were developed through a series of discoveries that led to important breakthroughs in biotechnology. Some of the pioneering scientists who made these discoveries received Nobel Prizes in Physiology or Medicine in 1978 and Chemistry in 1980 and 1993. (The Nobel Prize is the highest distinction awarded to scientists in these fields from around the world.) The work that you are about to do is based on this Nobel Prize-winning science—science that is significant and will continue to play an important role in the development of biotechnology and medicine. You will follow in the footsteps of the many scientists who have pushed and continue to push the boundaries of biotechnology. There are many advances still to be made—and students who decide to continue studying this field may contribute to those advances.

In science, the ability to keep track of what you are doing and communicate about your work is extremely important. To demonstrate that you performed an experiment, either so that it can be duplicated and verified by others or if you want to apply for a patent—you need to have a very accurate record of what you've done. As you carry out this program, carefully record your notes, ideas, observations, results, and answers to questions in a science notebook, in pen. (For scientific purposes, it is important to keep a record—even of your mistakes.) If possible, use a separate bound composition notebook and organize the labs with a table of contents at the front. Since you will use a pen to write with, you'll need to cross out any mistakes you make—and it is good practice to simply "X" out the section you want to change (so that it can still be read) and to note

why you've done so. Following these best practices will make this program even better preparation for you!

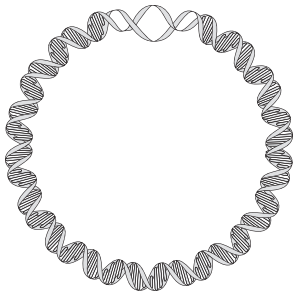
The Amgen Biotech Experience (formerly Amgen-Bruce Wallace Biotechnology Lab Program) had humble beginnings 30 years ago with visionary scientists and teachers who shared passion and energy for imparting their knowledge with students. Bruce Wallace, one of Amgen's first staff members, wanted all students to experience the joy of discovery and the excitement of having science at their fingertips. A desire for more robust science education at schools near Amgen's global headquarters led to involving area high school teachers and, later, a college professor, in developing curriculum and educator training in biotechnology. The program grew through word of mouth and teacher interest, and expanded over time to other states and countries.

Visit the ABE website at www.amgenbiotechexperience.com.



PROGRAM INTRODUCTION

AMGEN BIOTECH EXPERIENCE



WHAT IS BIOTECHNOLOGY?

At its simplest, biotechnology is the use of biological systems to create products. The use of yeast to make bread is one of the earliest examples of humans using a biological process (fermentation by yeast) to create a desired product (food).

It was not until the 1970s that the science of biotechnology really took off when scientists made two key discoveries about bacteria. The first discovery was that bacteria contain tiny circles of ***DNA*** (deoxyribonucleic acid, a double-stranded biomolecule that encodes genetic information), called ***plasmids*** within them. The second was that bacteria also contain ***proteins*** (large biomolecules that carry out essential functions in cells) called ***restriction enzymes*** that can cut DNA at very specific places.

The findings made by basic research often lead to fundamental understandings about the nature of life. In some instances, these findings can also lead to new tools and technologies that can improve life. With the discovery of plasmids and restriction enzymes, for example, a whole new era of biotechnology using recombinant DNA technology was launched. ***Recombinant DNA*** refers to DNA that contains sequences or genes from two or more sources—sometimes even from two different species! By harnessing natural biological processes, scientists can generate products that can contribute to human society in ways never before imagined.

Modern biotechnology is now used to develop hundreds of products and technologies—to create fuels to power the world, to develop better systems for the production of food, and to improve human health.

STUDYING HUMAN BIOLOGY TO TREAT DISEASES

Biopharmaceutical (biopharma) researchers study human biology to better understand how to develop solutions to improve the lives of people who suffer from serious diseases. To do so, these researchers study a disease closely,

exploring its mechanisms and the changes it causes to the human body. Based on this research, scientists can develop biopharmaceutical therapies that take advantage of biological systems to treat or cure these diseases.

The biopharma industry ushered in a new wave of protein-based medicines that are made through the marriage of science and the molecular machinery of ***cells*** (the basic units of any living organism that carry on the biochemical processes of life). The earliest biotech drugs were genetically engineered versions of human proteins—large molecules far too intricate to assemble through chemical processes but which could be made by harnessing cells with strategically-engineered DNA. Today, protein engineers can reconfigure nature's building blocks to design innovative structures that fight disease in a more sophisticated manner.

What is the relationship between DNA and proteins? Both are ***biomolecules***, large molecules made by living cells. When scientists investigated ***traits*** (genetically determined characteristics) in organisms, they found that proteins were responsible for traits and that DNA was responsible for creating proteins. For example, consider a plant that has the trait of red flowers. The flowers' red pigment is produced by the action of an ***enzyme*** (a protein that increases the rate of a chemical reaction). The DNA in that plant contains instructions for making proteins, including that enzyme. The part of a DNA molecule that has the instructions for making a particular protein is called a ***gene***.

THE FUTURE OF BIOPHARMA

With our advanced understanding of the human genome and the wealth of human genome data available today, biopharma researchers are finding new ways to identify the genetic basis of diseases and individual responses to treatments so that they can target therapies to specific people. Doctors can identify patients for whom certain medicines are ineffective because of their genetic profile, and instead choose options that will work better for that individual. The examination of the human genome and its variations allows researchers to better understand the disease-related genetic differences of diverse populations of people and then use that understanding to develop better medicines.

Biopharma researchers are also working on developing new mechanisms for treating disease. New "targeted" cancer drugs, for example, hold tremendous promise within the biotech industry. Chemotherapy drugs—the traditional treatment for cancer—target and destroy rapidly dividing cells. Unfortunately, these drugs often cause significant "collateral damage" because they are unable to differentiate between cancerous rapidly-dividing cells and normal rapidly-dividing cells. Chemotherapy can destroy healthy blood cells, hair follicles, and the cells lining the stomach and digestive tract, causing patients experience

debilitating side effects from these medications. Researchers are working hard to create drugs that will effectively eliminate cancerous cells but spare healthy tissues. Doctors are especially optimistic about the future of several recently developed immunotherapy drugs, which allow a patient's own immune system to fight their cancer. One such drug is a type of synthetic antibody, which is attracted only to proteins located on tumor cells. Once attached to a tumor cell, these antibodies release several proteins that both induce programmed cell death (apoptosis) and cause the cell to burst. The ability to selectively eliminate cancerous cells without damaging healthy cells would be an enormous step forward in treating cancer.

The field of ***genetic engineering*** (the process of altering the genetic material of cells or organisms to enable them to make new substances or perform new functions) that began in the 1970s has revolutionized medicine. With each passing year, the pace of discovery quickens and our understanding of the role of genetics in human health grows. Technology that allows us to quickly and efficiently edit DNA is being applied to the development of 42 new pharmaceuticals and is even being explored as a way to replace defective genes in human somatic cells—for example, to replace a defective gene that causes cystic fibrosis with a functional gene. Another recent advance allows researchers to reprogram adult cells into embryonic stem cells and then induce those cells to become any type of cell. These cells can be used to make model organs on which drugs can be tested outside of the human body. These technologies, and others that haven't yet been envisioned, are changing the future of medicine and providing dramatic improvement in human health and disease treatment.

DID YOU KNOW?

The DNA Code

DNA information is encoded by the arrangement of ***nucleotides***, small molecules that join together to form the DNA molecule. A DNA molecule has millions of nucleotides. There are four different kinds of nucleotides, and they are arranged in a specific ***sequence*** (order). A specific sequence of nucleotides in the DNA (i.e., a gene) is a code for how to make a specific protein. Think of a sequence of nucleotides as similar to a sequence of written musical notes—the code for how to play music. Just as different sequences of notes encode different songs, different sequences of nucleotides encode different proteins.

THE TOOLS AND TECHNIQUES OF BIOTECHNOLOGY

For the next few days, you will explore the science of biotechnology and the tools used by scientists to create products. Your first task is to try out two of the tools used in biotechnology, the micropipette and gel electrophoresis.

When carrying out any scientific experimentation, you will find that accuracy and precision are important as is ensuring that you follow procedures carefully. Throughout your experience with ABE, your goal should be to learn about how and why the tools and techniques you're learning are used.

USING THIS STUDENT GUIDE

Icons are used throughout the Student Guide to draw attention to various aspects of the curriculum. The following is a list of those icons and their meanings.

Icon	Meaning
	**DID YOU KNOW?**: Background information about concepts covered in the chapter.
	**STOP AND THINK**: Questions about the lab protocols.
	**CONSIDER**: Questions about important biological concepts.
	**SAFETY**: Reminders of key lab safety techniques.
	**LAB TECHNIQUE:** Useful lab techniques to improve efficiency and results.

PROGRAM INTRODUCTION GLOSSARY

Biomolecule: A molecule produced by living cells. Examples include proteins, carbohydrates, lipids, and nucleic acids.

Cells: The basic units of any living organism that carry on the biochemical processes of life.

DNA (deoxyribonucleic acid): A double-stranded biomolecule that encodes genetic information.

Enzyme: A protein that increases the rate of a chemical reaction.

Gene: The part of a DNA molecule that contains the instructions for making a particular protein.

Genetic engineering: A branch of biotechnology that uses specific procedures and techniques to change an organism's DNA.

Nucleotides: Small molecules that join together to form the DNA molecule.

Plasmid: A circular molecule of DNA.

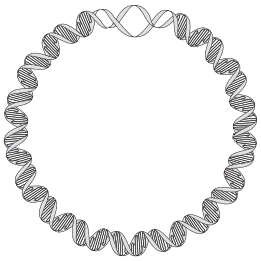
Protein: A large biomolecule. Proteins carry out essential functions in cells, from forming cellular structures to enabling chemical reactions to take place.

Recombinant DNA: DNA that contains sequences or genes from two or more sources.

Restriction enzyme: A protein that can cut DNA at a specific sequence.

Sequence: A set of related events, movements, or items (such as nucleotides) that follow each other in a particular order.

Trait: A genetically determined characteristic. DNA codes for proteins, which determine traits.



CHAPTER 1

SOME TOOLS OF THE TRADE

INTRODUCTION

The year 1978 marked a major breakthrough in medicine. For the first time ever, scientists were able to engineer bacteria capable of producing human proteins. They achieved this by strategically inserting small pieces of human DNA into bacterial cells. This new technology, termed genetic engineering, can be used to make proteins that treat the symptoms of certain ***genetic diseases*** (those caused by a change in DNA, often inherited from parents). Genetic engineering, also called genetic modification, is the direct manipulation of an organism's genes using biotechnology.

To carry out genetic engineering, you need good laboratory skills. In this chapter, you'll focus on gaining practice in the use of ***micropipettes*** (instruments used to transfer small volumes of liquid) and ***gel electrophoresis*** (a technique for separating and identifying biomolecules)—two critical skills for biotechnology. You will complete two labs, using instruments and supplies that are identical to the ones used in biotechnology research laboratories. These labs are the first step in building the skills you'll need to be successful in biotechnology.

CHAPTER 1 GOALS

By the end of this chapter, you will be able to do the following:

- Correctly use micropipettes and the technique of gel electrophoresis
- Explain the importance of micropipettes and gel electrophoresis in genetic engineering
- Describe how gel electrophoresis separates DNA
- Explain how genetic engineering can be used to treat some genetic diseases

WHAT DO YOU ALREADY KNOW?

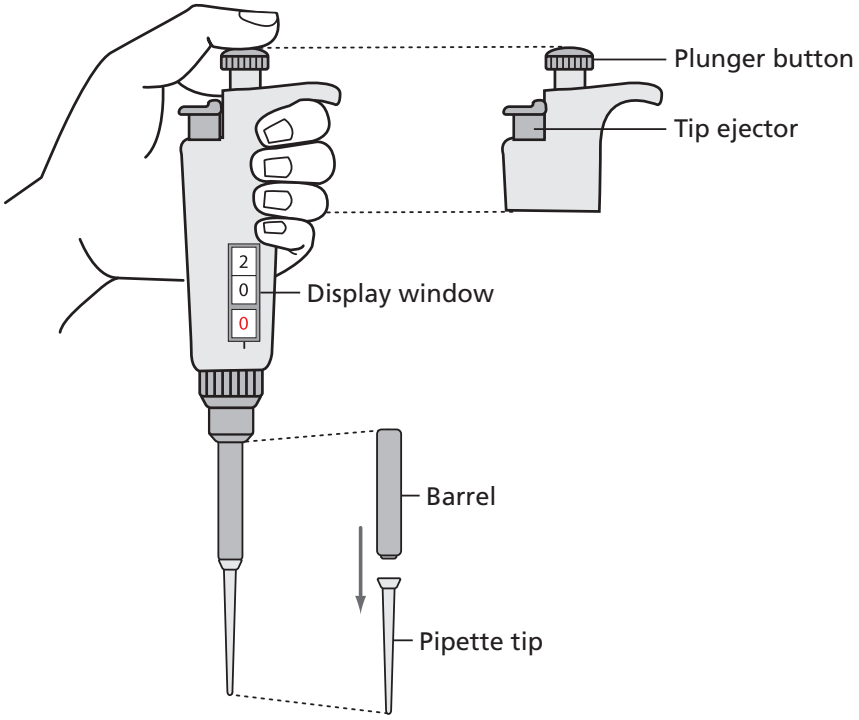
Discuss the following questions with your partner, and write your ideas in your notebook. Be prepared to discuss your responses with the class. Don't worry if you don't know all the answers. Discussing these questions will help you think about what you already know about biotechnology.

1. What tools and techniques of biotechnology have you used before? What did you use them for?
2. Why is precision important when you are carrying out biotechnology procedures?

LABORATORY 1.1: HOW TO USE A MICROPIPETTE

The purpose of this laboratory is to introduce you to an important tool used in genetic engineering: the micropipette, shown in **Figure 1.1**. A micropipette is used to transfer very small and exact volumes of liquids in either milliliters (mL, thousandths of a liter) or microliters (µL, millionths of a liter), which are the measurements of volume most often used in genetic engineering. This laboratory will give you the chance to learn how to use the micropipette and to see the relative size of different amounts of solution measured by this very precise tool and how precise the amounts that you can measure with it are.

Figure 1.1: A P-20 micropipette



BEFORE THE LAB

Respond to the following with your group and be prepared to share your responses with the class.

1. Why do you think it is necessary to use very small and exact volumes of material in biotechnology?
2. Read through the *Methods* section on pages 17 through 19 and briefly outline the steps, using words and a flowchart.

MATERIALS

Reagents

- A plastic microfuge tube rack with a microfuge tube of red dye solution.

Equipment and Supplies

- P-20 micropipette (measures 2.0–20.0 µL)
- Tip box of disposable pipette tips
- Laminated micropipette practice sheet
- Waste container for used tips and microfuge tubes (will be shared among groups)

SAFETY:

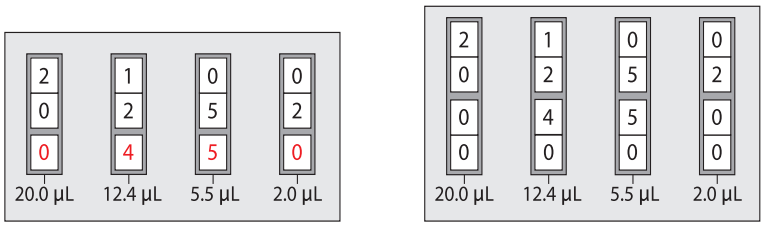
- **All appropriate safety precautions and attire required for a science laboratory should be used, including safety goggles. Please refer to your teacher's instructions.**
- **Wash your hands well with soap after completing the lab.**

METHODS

1. Check your rack to make sure that you have the reagent listed in *Materials*.
2. Review the parts of the micropipette shown in (see **Figure 1.1** on page 16).
3. Find the display window on the handle of the micropipette.
4. Different micropipettors are adjusted in different ways. Many have a wheel that adjusts the volume. Others are adjusted by turning the plunger. In most cases, you turn right to increase the volume and left to decrease it.
5. **Figure 1.2** shows four micropipette volumes. Practice setting the micropipette to these volumes.

   LAB TECHNIQUE: Never set the P-20 micropipette lower than 2.0 µL or higher than 20.0 µL or you could damage the equipment.

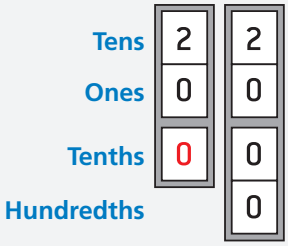
Figure 1.2: Four volumes shown on two different P-20 micropipettors



The display window of a micropipette shows how much fluid it will load and dispense. Four examples of displays and the corresponding amounts are shown.

6. Review the laminated micropipette practice sheet. Each group member will pipette five drops of different volumes onto the sheet. Pipetting consists of two parts: loading the liquid into the micropipette, and dispensing the liquid from the micropipette.

P-20 Micropipette



7. Load the micropipette with 20.0 µL of red dye (RD) by doing the following:

   a. Set the micropipette to 20.0 µL.
   b. Open the tip box. Lower the micropipette onto a tip and press down firmly (do not touch the tip with your fingers). Close the box when done.
   c. Bring the micropipette and the RD tube to eye level.
   d. Use your thumb to press the plunger to the first stop position, which is your first point of resistance.

LAB TECHNIQUE: When loading the micropipette, only press the plunger to the first stop or you will draw too much solution into the pipette tip.

   e. Put your pipette tip into the RD and slowly release the plunger to draw up the solution.

LAB TECHNIQUE: Do not lay down a micropipette with fluid in the tip or hold it with the tip pointed upward. If the disposable tip is not firmly seated onto the barrel, fluid could leak back into the pipette.

8. Dispense RD onto the laminated sheet by doing the following:

   a. Place the pipette tip over the 20.0 µL circle.
   b. Use your thumb to press the plunger to the first stop position and then press down to the second stop.

LAB TECHNIQUE: When dispensing liquid from the micropipette, press the plunger to the first stop to dispense most of the liquid and then press the plunger to the second stop in order to dispense the last little bit.

   c. With the plunger still depressed, pull the pipette away from the paper—this prevents you from accidentally pulling the liquid back into the tip.

9. Without setting down the micropipette, twist the plunger button to set it to 15.0 µL and repeat steps 7b–8c, dispensing over the 15.0 µL circle.
10. Without setting down the micropipette, set it to 10.0 µL and repeat steps 7b–8c, dispensing it over the 10.0 µL circle when dispensing the liquid.
11. Without setting down the micropipette, set it to 5.0 µL and repeat steps 7b–8c, dispensing it over the 5.0 µL circle.
12. Without setting down the micropipette, set it to 2.0 µL and repeat steps 7b–8c, dispensing it over the 2.0 µL circle.
13. Use the tip ejector to place your pipette tip into the waste container.

STOP AND THINK:

- **When loading or dispensing a solution, why is it important to actually see the solution enter or leave the pipette tip?**
- **You were instructed to avoid contact with the pipette tips—for example, you were asked to put the pipette tip on without using your hands, to avoid setting down the micropipette, to use the ejector button to remove the tip, and to keep the tip box closed. If you were working with plasmids and bacterial cells, why would these precautions be important?**

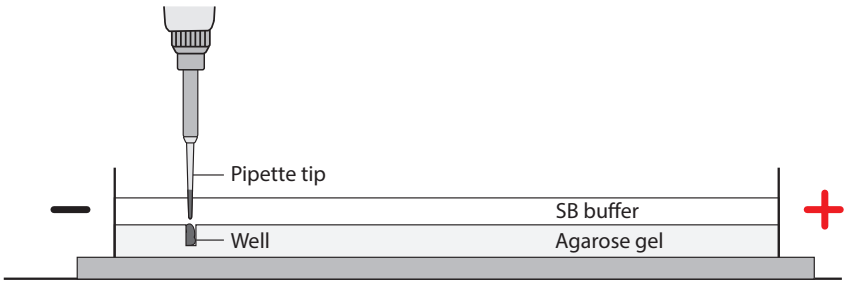
14. Using the micropipette practice sheet, each person in your group should have a chance to load and dispense the five drops of different volumes, with each person using a new pipette tip.
15. When everyone in your group has had a chance to dispense RD onto the micropipette practice sheet, draw the approximate sizes of each drop in your notebook (or take a photograph and tape it into your notebook) and label them with the amounts.

LABORATORY 1.2: GEL ELECTROPHORESIS

The purpose of this laboratory is to give you experience with gel electrophoresis, which is used to separate and identify a mixture of biomolecules including DNA; the size of the components of each mixture can then be identified by their location in the gel. Biomolecules are too small to see, and estimating their exact size is very difficult. Gel electrophoresis allows scientists to easily visualize information and compare various biomolecules. Gel electrophoresis works based on the fact that many biomolecules have a negative charge, which means that they will move in response to an electric charge. The biomolecules move through a gel, and the distance they travel varies primarily according to their size, although molecular shape and degree of charge also influence their movement.

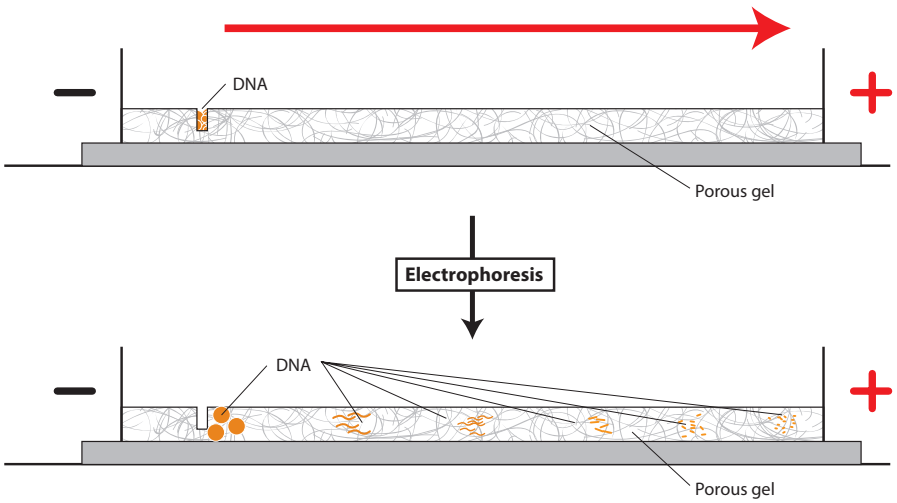
The electrophoresis setup consists of a box containing an agarose gel and two electrodes that create an electric field across the gel when the box is attached to a power supply. The negative electrode is black, and the positive electrode is red. ***Wells*** are depressions in the agarose that can hold small volumes of samples. The molecules in a sample will travel through the gel and be sorted based on their properties of charge and size. Samples of negatively-charged biomolecules are pipetted into wells near the negative (black) electrode. The samples move through the gel toward the positive (red) electrode, as shown in **Figure 1.3**, because opposite charges attract and like charges repel.

Figure 1.3: The gel electrophoresis unit



The gel that the biomolecules move through is composed of ***agarose***, a polysaccharide (complex sugar) found in seaweed. Its structure is a porous matrix (like a sponge) with lots of holes through which the solution and biomolecules flow. **See Figure 1.4**.

Figure 1.4: How biomolecules, including DNA, move through the agarose gel matrix in gel electrophoresis



BEFORE THE LAB

Respond to the following with your group, and be prepared to share your responses with the class.

1. In what circumstances might it be important to use gel electrophoresis to separate and identify plasmids and short linear pieces of DNA?
2. Read through the *Methods* section on pages 22 through 25 and briefly outline the steps for Part A and for Part B, using words and a flowchart.

MATERIALS

Reagents

- A plastic microfuge tube rack with the following:
  - Microfuge tube of red dye solution
  - Microfuge tube of dye solution 1 (S1)
  - Microfuge tube of dye solution 2 (S2)
  - Microfuge tube of dye solution 3 (S3)
- 50-mL flask containing 1x sodium borate buffer (1x SB buffer) (shared with another group)

Equipment and Supplies

- P-20 micropipette (measures 2.0–20.0 µL)
- Tip box of disposable pipette tips
- 2 pipetting practice plates loaded with 0.8% agarose gel

- Electrophoresis box loaded with 0.8% agarose gel (will be shared among groups)
- Microcentrifuge (will be shared among all groups)
- Waste container for used tips and microfuge tubes (will be shared among groups)

SAFETY:

- **All appropriate safety precautions and attire required for a science laboratory should be used, including safety goggles. Please refer to your teacher's instructions.**
- **Wash your hands well with soap after completing the lab.**

METHODS

PART A: PIPETTING INTO WELLS

You will practice pipetting RD into preformed wells in an agarose gel.

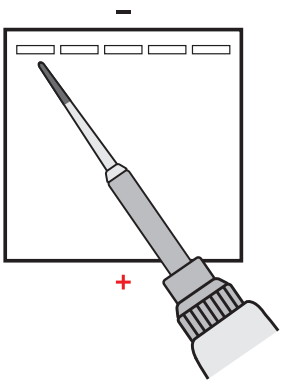
1. Check your rack to make sure that you have the red dye (RD) tube.
2. Fill the two pipetting practice plates with 1x SB buffer to a level that just covers the entire surface of the gel. If you see any "dimples" over the wells, add more ***buffer*** (a solution that can maintain a nearly constant pH. In gel electrophoresis, it prevents the gel's pH from changing due to the electrical current).
3. Set the P-20 micropipette to 10.0 µL and put on a pipette tip.
4. Draw up 10.0 µL of RD in to the pipette.

   LAB TECHNIQUE: Do not lay down a micropipette with fluid in the tip or hold it with the tip pointed upward.

5. Dispense RD into a well in one of the practice plates by doing the following:
   a. Place your elbow on the table to steady your pipette hand. If needed, also use your other hand to support your pipette hand.
   b. Lower the pipette tip until it is under the buffer but just above the well.

   LAB TECHNIQUE: Be careful not to place your pipette tip into the well or you might puncture the gel, which will make the well unusable.

   c. Gently press the plunger to slowly dispense the sample. To avoid getting air into the buffer, do not go past the first stop. The sample will sink into the well.



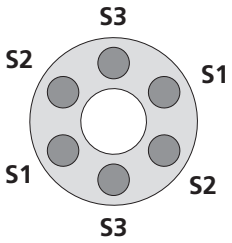
6. Repeat steps 4 and 5 until all the practice plate wells have been filled. Everyone in your group should get an opportunity to practice pipetting into the wells.
7. Eject the pipette tip.

PART B: SEPARATING DYES WITH GEL ELECTROPHORESIS

Now you will use gel electrophoresis to separate different dyes. First you will add dyes into wells in the gel electrophoresis unit. You will then turn the unit on in order to move the negatively charged dyes through the gel. (You will share the electrophoresis boxes with one other group; your teacher will tell you which wells your group should use.)

1. Check your rack to make sure that you have the three dye solutions (S1, S2, and S3).
2. Review **Figure 1.4** on page 21. Check to make sure that the wells in the gel are located near the negative (black) electrode.
3. Fill the box with 1x SB buffer to a level that just covers the entire surface of the gel. If you see any "dimples" over the wells, add more buffer.
4. Centrifuge the S1, S2, and S3 tubes.

LAB TECHNIQUE: **Distribute the tubes evenly in the microcentrifuge so that their weight is balanced.**



5. Make a drawing in your notebook that shows the location of the wells in the electrophoresis box. Record which solution you will place in each well.
6. Set the P-20 micropipette to 10.0 µL and put on a pipette tip.
7. Draw up 10.0 µL of S1 into the pipette.
8. Dispense the S1 into the well you've designated for that solution by doing the following:
   a. Place your elbow on the table to steady your pipette hand. If needed, also use your other hand to support your pipette hand.
   b. Lower the pipette tip until it is under the buffer but just above the well.

LAB TECHNIQUE: **Do not puncture the gel with the pipette tip or it will become unusable; the sample will sink into the hole below the gel instead of moving through the gel.**

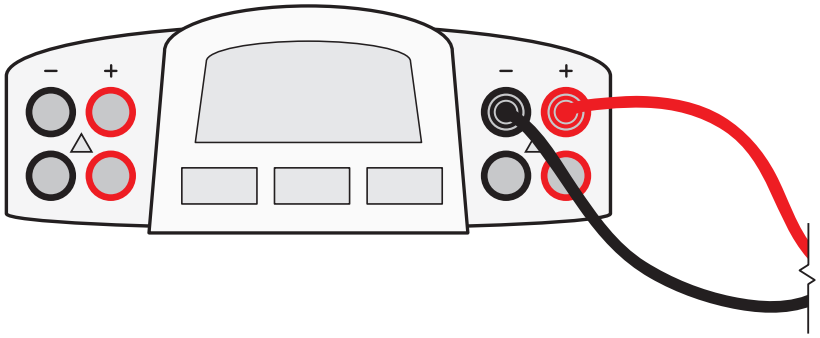
c. Gently press the plunger to slowly dispense the sample. To avoid getting air into the buffer, do not go past the first stop. The sample will sink into the well.

LAB TECHNIQUE:

- **While the plunger is still depressed, pull the tip out of the buffer so that you don't draw up the solution or buffer.**
- **Use a fresh pipette tip for each sample.**

9. Using a new pipette tip with each solution, repeat steps 7 and 8 for S2 and S3.
10. When all the samples have been loaded, close the cover tightly over the electrophoresis box. (Carefully close the cover so that samples don't spill.)
11. Connect the electrical leads to the power supply. Connect both leads to the same channel, with cathode (–) to cathode (black to black) and anode (+) to anode (red to red). See **Figure 1.5**

Figure 1.5: Leads from electrophoresis box connected to correct channel in power supply



12. Turn on the power supply and set the voltage to 130–135 V. (You will see bubbles form in the buffer at the red [+] end of the electrophoresis unit.)
13. After two or three minutes, check to see if the dyes are moving toward the positive (red) electrode. You should begin to see the purple dye (bromophenol blue) beginning to separate from the blue dye (xylene cyanole).

STOP AND THINK:

- **Study your gel electrophoresis results. Which solution sample contained a single dye: S1, S2, or S3? How do you know?**
- **What electrical charge do the dyes have? Explain your reasoning.**
- **The dyes that you are separating are orange G (yellow), bromophenol blue (purple), and xylene cyanole (blue). If the molecular shape and electric charge of all three dyes are similar, what is the order of the dyes from heaviest to lightest molecules, based on your initial results? Why do you think this is the correct order?**

14. In approximately 10 minutes, or when you can distinguish all three dyes, turn off the power switch and unplug the electrodes from the power supply. Do this by grasping the electrode at the plastic plug, NOT the cord.
15. Carefully remove the cover from the gel box, and observe the dyes in the gel.
16. In your notebook, draw the relative location of the bands and their colors in each of the lanes containing your samples.
17. Leave the gels in the gel box.

CHAPTER 1 QUESTIONS

1. Why would it be beneficial to use a micropipette to measure reagents in biotechnology rather than another measuring instrument?
2. What do the results of gel electrophoresis tell you about genetic material?

DID YOU KNOW?

Gel Electrophoresis in DNA Fingerprinting

DNA fingerprinting uses gel electrophoresis to distinguish between samples of genetic material. In DNA fingerprinting, human DNA molecules are treated with enzymes that chop them at certain characteristic points, thereby reducing the DNA to a collection of smaller and more manageable pieces. The DNA fragments are loaded into a gel and placed in an electrical field, which sorts the DNA fragments into various bands. These bands can be colored with a radioactive dye to make them visible to imaging techniques. Methods of DNA identification have been applied to many branches of science and technology, including medicine (prenatal tests, genetic screening), conservation biology (guiding captive breeding programs for endangered species), and forensic science. In the latter discipline, analysis of the pattern of DNA fragments that results from the action of restriction enzymes enables us to discriminate between suspects accused of a crime or between potential fathers in a paternity suit.

CHAPTER 1 GLOSSARY

Agarose: A polymer made up of sugar molecules that is used as the matrix in gel electrophoresis procedures.

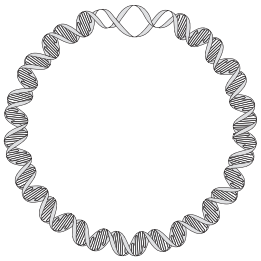
Buffer: A solution that can maintain a nearly constant pH. In gel electrophoresis, it prevents the gel's pH from changing due to the electrical current.

Gel electrophoresis: The movement of charged molecules toward an electrode of the opposite charge; used to separate biomolecules. When used to separate DNA fragments, electrophoresis will separate the fragments by size, with smaller fragments moving faster than larger fragments.

Genetic disease: Those diseases caused by a change in DNA. Genetic diseases are often inherited from parents.

Micropipette: A laboratory instrument used to measure, dispense, and transfer very small amounts of liquid.

Well: A depression in the agarose that can hold small volumes of samples.



CHAPTER 2A

HOW DO YOU BEGIN TO CLONE A GENE?

INTRODUCTION

In the Program Introduction, you learned about the development of biopharmaceuticals and were introduced to the techniques used in developing these therapeutics. One of these techniques—bacterial transformation—allows human genes to be inserted into bacteria, enabling the bacteria to produce the human therapeutic proteins. Chapter 1 gave you a chance to work with two physical tools and techniques of genetic engineering that are used to clone a gene: the micropipette and gel electrophoresis. In this chapter you will work with two other important genetic engineering tools—plasmids and restriction enzymes. These "tools" are actually biomolecules found in many bacteria, and their discovery was crucial to genetic engineering. With these tools, scientists can modify microorganisms to make human proteins. You will now learn more about these tools and will then carry out the first steps in your quest to clone a gene.

CHAPTER 2A GOALS

By the end of this chapter, you will be able to do the following:

- Describe the characteristics of plasmids
- Explain how plasmids are used in cloning a gene
- Describe the function of restriction enzymes
- Explain how to use restriction enzymes to create a recombinant plasmid

WHAT DO YOU ALREADY KNOW?

Discuss the following questions with your partner and write your ideas in your notebook. Be prepared to discuss your responses with the class. Don't worry if you don't know all the answers. Discussing these questions will help you think about what you already know about DNA, plasmids, and restriction enzymes.

1. What is the structure and function of DNA? Describe in words or a drawing the structure of a DNA molecule. Be as detailed as possible.

2. All living organisms contain DNA. In what ways is the DNA from different organisms the same, and in what ways does it vary?

3. Why it is possible for a bacterial cell to make a human protein from the instructions encoded in a human gene? Explain your answer, using your understanding of genes and how they are expressed.

4. As described in the Program Introduction, scientists use two biological tools to engineer organisms to make new proteins: plasmids and restriction enzymes. What do you remember about how these tools are used?

YOUR CHALLENGE

Now that you've explored some of the basic tools used in biotech, you will have the opportunity to carry out some of the same procedures that scientists use to produce human therapeutic proteins. But instead of producing human protein, you will engineer ***E. coli***—a common bacterium found in the gut of warm blooded animals—to produce a sea anemone protein called ***red fluorescent protein*** (RFP), which is directed by a gene called *rfp*. A sea anemone is a soft-bodied animal related to coral and jellyfish. In the laboratory, you will give *E. coli* a new protein that will give it a trait it did not have before: the ability to glow. How will you know if you are successful? The bacteria you create will have a new and highly visible trait: They will now produce RFP, which will make the cells appear red or bright pink!

NOTE: The number of steps will vary depending on how much time your class has available.

DID YOU KNOW?

Red Fluorescent Protein in Sea Anemones

RFP is derived from a protein found in sea anemones (see **Figure 2A.1**). While sea anemones are sedentary, remaining attached to rocks, they are also predatory animals, using their stinging tentacles to catch their prey. The protein glows because it can absorb one color of light and then emit light of a different color—a process known as ***fluorescence***. But why is it important for sea anemones to fluoresce? Our best guess is that fluorescent proteins help sea anemones survive, but the role these proteins play is not yet well-understood.

Fluorescent molecules may serve as a sunblock, turning harmful UV light into light that is less damaging to the anemone's tissues. Another possibility is that while humans can't detect the fluorescence in bright sunlight, some animals may be able to, causing prey to be attracted to the glow.

Figure 2A.1: The sea anemone, *Discosoma* sp.

BEGINNING TO CLONE A GENE

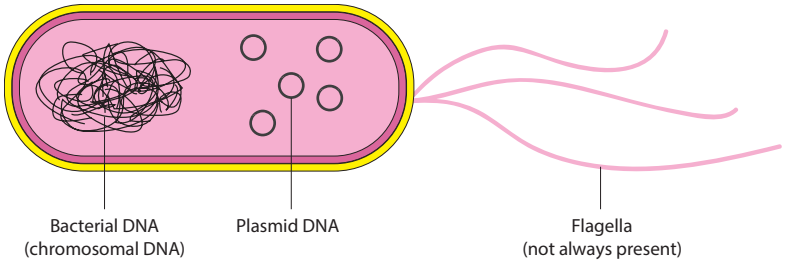
In this chapter, you explore the use of plasmids and restriction enzymes as tools of biotechnology. ***DNA cloning*** is the process of making many exact copies of a particular piece of DNA. First, a specific gene (for example, a gene for a human therapeutic protein) is cut from its source, using a restriction enzyme. It is then pasted together with other fragments to create a ***recombinant plasmid***, a plasmid built with fragments of DNA from different sources.

The discovery of plasmids and restriction enzymes in bacteria is a classic example of how findings from basic research can revolutionize a field. With the discovery of these biomolecules, scientists made major breakthroughs in understanding fundamental processes of life and in developing life-improving products.

PLASMIDS

Many different types of bacteria carry two forms of DNA: (1) a single chromosome made up of a large DNA molecule that contains all the information needed by the organism to survive and reproduce, and (2) plasmids, small circular DNA molecules, ranging in size from 1,000 to 200,000 ***base pairs*** (two nitrogenous bases joined to connect complementary strands of DNA) that are present in multiple copies separate from the chromosomal DNA (see **Figure 2A.2**). Some bacteria carry as many as 500 plasmids in each cell.

Figure 2A.2: DNA in bacterial cells



Four characteristics of plasmids make them ideal ***vectors*** (vehicles for carrying DNA sequences from one organism to another) for genetic engineering: (1) the ability to replicate; (2) the ability to initiate transcription; (3) a gene or genes that code for resistance to ***antibiotics***, a class of compounds that kill or inhibit the growth of microorganisms; and (4) the ability to be passed between bacteria. These characteristics are described in detail below:

1. Plasmids have the ability to replicate, that is, to make copies of themselves independently of the bacterial chromosome. To do this, plasmids include a specific sequence to which the host cell DNA synthesis enzymes bind and initiate ***DNA replication*** (a biological process that occurs in all living organisms to make copies of their DNA). This sequence is called the ***origin of replication (ori)*** site.

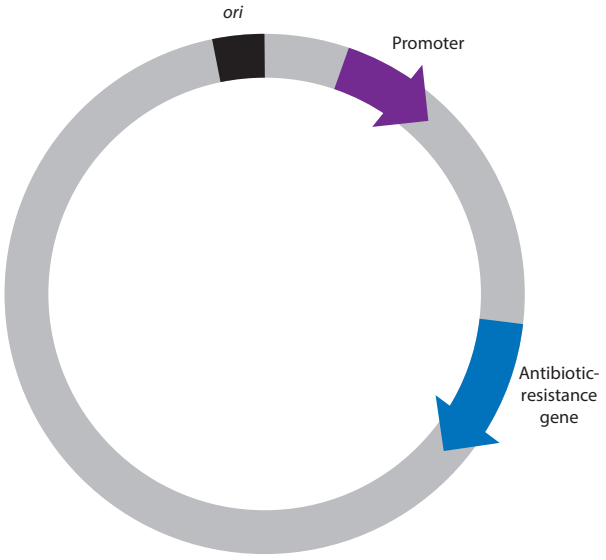
2. Plasmids have the ability to initiate ***transcription***—the process by which information encoded in DNA is transferred to ***messenger RNA (mRNA)***. mRNA is an RNA molecule transcribed from the DNA of a gene and used as the template for protein synthesis, using the host cell ***RNA polymerase*** (a protein that makes mRNA from DNA). ***RNA***, or ***ribonucleic acid***, is a single-stranded biomolecule made up of a nitrogenous base, a ribose sugar, and a phosphate; it plays a critical role in protein synthesis, transmitting genetic information from DNA to the ribosome where proteins are then made. This ability requires another sequence, called the ***promoter*** (a specific DNA sequence that binds RNA polymerase and initiates transcription of the gene). The promoter binds RNA polymerase, and this is where transcription is initiated. All genes have promoters located next to them in the DNA. For human therapeutic protein genes to be expressed in bacteria, they must be inserted into the plasmid next to the promoter.

3. Plasmids possess a gene or genes that code for ***antibiotic resistance*** (the state in which bacteria are no longer sensitive to an antibiotic and will continue to grow and divide in the presence of that antibiotic). These genes code for proteins that inhibit the action of antibiotics secreted by microorganisms, which can confer a selective advantage in nature to plasmid-containing bacteria in a microbial population where bacteria compete for survival.

4. Plasmids can be passed on from one bacterial strain to another in a process called ***bacterial conjugation***, which enables bacteria to share and exchange genetic information. When a plasmid with a gene for antibiotic resistance is taken in by bacteria lacking that plasmid, the bacteria will then become resistant to that specific antibiotic. In nature, conjugation occurs with very low efficiency—that is, only a small percentage of bacteria in a population can take in plasmid DNA at any point in time.

Figure 2A.3 illustrates the basic components of a plasmid.

Figure 2A.3: A plasmid vector



CONSIDER: Use what you know about natural selection and evolution to describe how plasmids might confer a selective advantage to their host bacteria.

In developing techniques for cloning genes in bacteria, scientists had a powerful tool in plasmids—a vector that can be taken in by bacteria, that replicates in bacteria to produce many copies of itself, that has a promoter for transcription of an inserted gene, and that carries a gene for antibiotic resistance. The presence of an antibiotic-resistance gene on the plasmid vector allows scientists to identify the small percentage of bacteria that took in the plasmid. Bacteria that did not take in the plasmid will be killed by the antibiotic. Those that have the plasmid with the gene of interest will survive and grow. If you carry out the lab in Chapter 5, you will take advantage of these features of plasmids when you transfer your recombinant plasmid into bacteria.

Once scientists recognized the power of plasmids as a potential vector, the next challenge was to determine how to incorporate a foreign gene of interest, such as the insulin gene, into the plasmid DNA. The plasmids you will work with in this and subsequent labs contain the genes for resistance to the antibiotics ampicillin and kanamycin. These genes produce proteins that inactivate the target antibiotic by chemically modifying its structure.

RESTRICTION ENZYMES

In the early 1950s, scientists observed that certain strains of *E. coli,* a common bacterium found in the human gut, were resistant to infection by ***bacteriophage***—a virus that infects bacteria by injecting its DNA into the cell and then commandeering the host cell's molecular processes to make more bacteriophage). Investigation of this primitive bacterial "immune system" led to the discovery of restriction enzymes, proteins that restrict the growth of bacteriophage by recognizing and destroying the phage DNA without damaging the host (bacterial) DNA. Subsequent studies demonstrated that restriction enzymes from different strains of bacteria cut DNA at specific sequences, which are called ***restriction sites***.

CONSIDER: How do bacteria that carry a restriction enzyme avoid cutting up their own DNA?

Table 2A.1 provides examples of restriction enzymes isolated from different strains of bacteria and the DNA sequences they cut. In the examples shown, the enzymes cut asymmetrically on the strands of DNA, leaving single-stranded overhanging sequences at the site of the cut. For example, a cut (or ***digestion***) with *Eco*RI will leave an AATT overhang (or "***sticky end***") on one strand and a TTAA sticky end on the other strand.

Table 2A.1: Restriction enzymes used in this laboratory

Source	Restriction enzyme	Restriction site
Escherichia coli	*Eco*RI	5′ G↓AATTC 3′ 3′ CTTAA↑G 5′
Bacillus amyloliquefaciens	*Bam*HI	5′ G↓GATCC 3′ 3′ CCTAG↑G 5′
Haemophilus influenzae	*Hin*dIII	5′ A↓AGCTT 3′ 3′ TTCGA↑A 5′

Note: The symbols ↑ and ↓ indicate where the DNA is cut.

CONSIDER:

- **What is the sequence of the sticky end that results when DNA is cut with *Bam*HI? With *Hin*dIII?**
- **Scientists can modify plasmids to have a single restriction enzyme site. Imagine that you have a plasmid with a single *Eco*RI site. Draw the structure of the plasmid after it has been cut with the enzyme, and show the nucleotide sequences left at the site of the cut. If you wanted to insert a gene from a plant at this site, what enzyme would you use to cut the plant DNA with? Explain your response.**

DID YOU KNOW?

The Rise of Antibiotic-Resistant Bacteria

Antibiotics and similar drugs have been used for the last 70 years to treat patients who have infectious diseases. When prescribed and taken correctly, antibiotics are enormously valuable in patient care. However, these drugs have been used so widely and for so long that the infectious organisms the antibiotics are designed to kill have adapted to them, making the drugs less effective. Antibiotic resistance occurs when some bacteria in a population are able to survive when exposed to one or more antibiotics. These species that have become resistant cause infections that cannot be treated with the usual antibiotic drugs at the usual dosages and concentrations. Some have developed resistance to multiple antibiotics and are dubbed multidrug-resistant bacteria or "superbugs."

Antibiotic resistance is a serious and growing phenomenon and has emerged as one of the major public health concerns of the 21st century. When drug-resistant organisms acquire resistance to first-line antibiotics (those selected on the basis of several advantages, including safety, availability, and cost), the use of second-line agents is required. These are usually broader in spectrum, may be less beneficial in relation to the associated risks, and may be more expensive or less widely available.

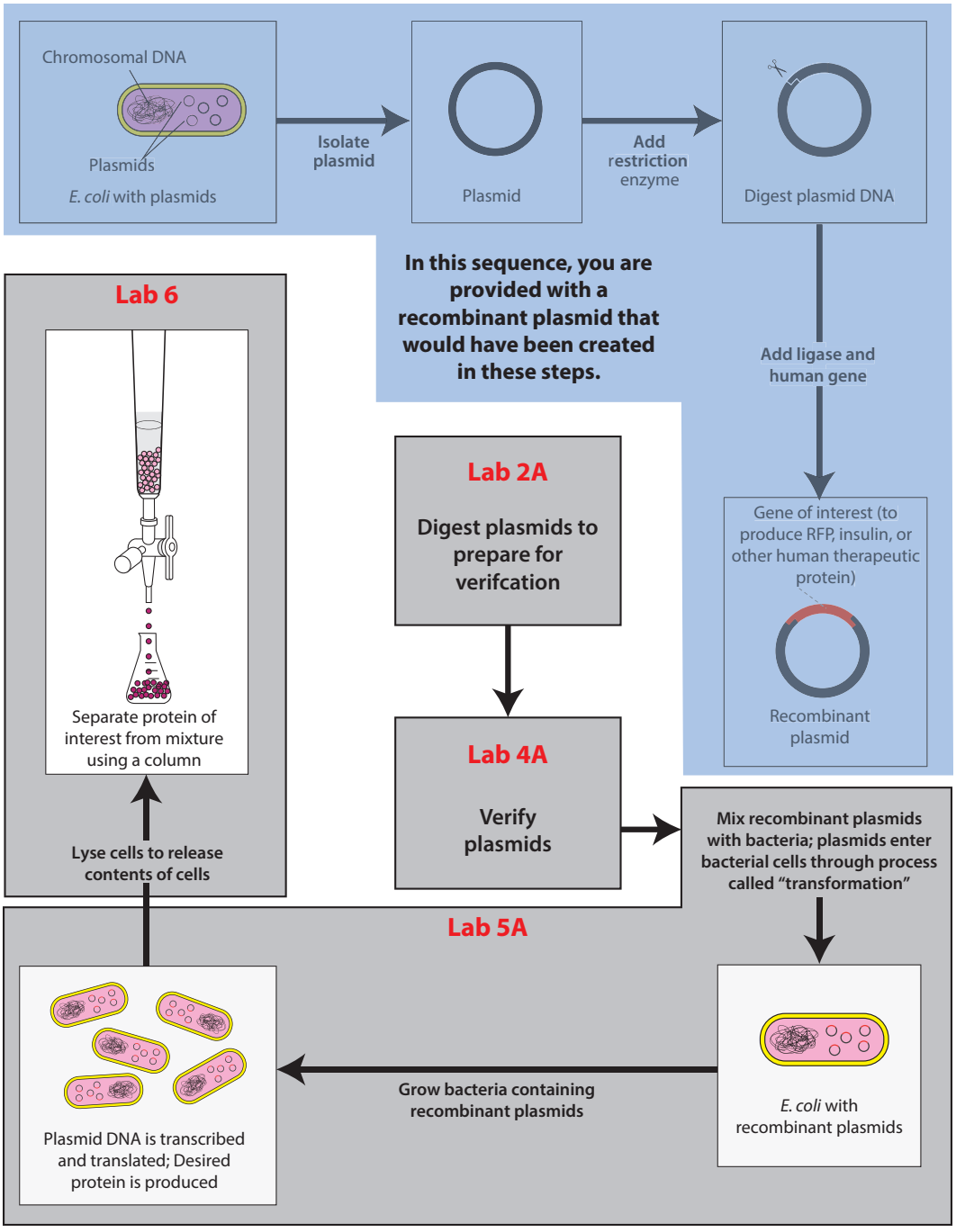
PRODUCING HUMAN THERAPEUTIC PROTEINS IN BACTERIA

Do you know somebody who has ***diabetes*** (a disease that occurs when a person's blood glucose [sugar] is too high), ***hemophilia*** (which occurs when the ability of blood to clot is reduced), and ***growth deficiency*** (a disease in which a person does not grow properly)? These three diseases result from the inability of a person's body to produce certain proteins. In diabetes, the body is unable to manufacture or produce ***insulin*** (a hormone produced in the pancreas that controls the amount of glucose in the blood). People with hemophilia are unable to make a ***blood clotting factor*** (a variety of proteins in blood plasma that participate in the clotting process). Growth deficiency is the result of the inability to make ***human growth hormone*** (a hormone secreted by the pituitary gland that stimulates growth). A patient with any of these diseases must be treated with the missing protein.

Prior to the development of recombinant DNA technologies, human therapeutic proteins were extracted from animals or other humans. Insulin was originally isolated from the pancreases of pigs and cows. Human growth hormone was extracted from the pituitary glands of human cadavers. These methods were effective, but using animal-produced proteins sometimes resulted in adverse reactions and making large enough quantities was difficult. Now, scientists have figured out how to add human DNA to bacterial DNA, allowing the bacteria to produce a human protein.

In the genetic engineering process, a human gene is added to a plasmid that has been cut using restriction enzymes. The plasmid is taken up by bacterial cells in a process called ***bacterial transformation***, and the cells make the human protein that is encoded by the human gene along with their own proteins (see **Figure 2A.4**). During this process, scientists use a combination of tools, some human-made and some biological. Throughout these labs, you're going to explore and use these tools so that you get a firsthand understanding of how they work.

Figure 2A.4: Making a human therapeutic protein in bacteria



CLONE THAT GENE

You now know about two biological tools for cloning a gene: plasmids and restriction enzymes.

1. Plasmids have several important features:
    - A sequence for the initiation of DNA replication, called the *ori* site, which allows the plasmid to replicate in the bacteria using the host DNA synthesis enzymes
    - A promoter for initiating transcription of the inserted gene
    - A gene encoding a protein for antibiotic resistance, which allows for identification of bacteria that have taken in the plasmid
2. Restriction enzymes digest both the plasmid and the human DNA containing the gene of interest (such as insulin) to be cloned.

How do scientists use these two tools to create a recombinant plasmid, which contains the human gene inserted into a bacterial plasmid? One important step is choosing a restriction enzyme (or enzymes) that cuts the plasmid and the human DNA. The restriction enzyme(s) must do all of the following:

- Cut the plasmid at a site (or sites) that allows for the insertion of the new gene.
- Cut the plasmid at an appropriate site to ensure that no important genes or sequences are disrupted, including the *ori* site, the promoter, and at least one of the genes encoding antibiotic resistance.
- Cut the plasmid near the promoter so that the inserted gene can be expressed.
- Cut the human DNA as close as possible to both ends of the gene of interest so that it can be inserted into the appropriate site in the plasmid DNA, without cutting within the gene.

STOP AND THINK: Why is it important that the same enzyme or enzymes be used to cut both the plasmid and the gene of interest from the human DNA?

In this activity, you will make a paper model of a recombinant plasmid that contains a gene for a human therapeutic protein—in this case, insulin. You have three tasks:

1. Cut the plasmid and the human DNA with the appropriate restriction enzyme
2. Insert the insulin gene into the plasmid DNA
3. Determine which antibiotic you would use to identify bacteria that have taken in the plasmid

HANDOUTS

- **Plasmid Diagram (RM 2)**
- **Human DNA Sequence (RM 3)**

PROCEDURE

1. On the **Plasmid Diagram (RM 2)**:
    - Use scissors to cut out the plasmid sequence, and tape the ends together to make a paper model of the plasmid.
    - Locate the positions of the *ori* site, the promoter, and the genes for antibiotic resistance.
    - Locate the positions of each restriction enzyme restriction site.

2. Choose the restriction enzyme that should be used to cut the plasmid. Verify that the restriction enzyme meets all the following criteria:
    - It leaves the *ori* site, the promoter, and at least one antibiotic-resistance gene intact.
    - It cuts the plasmid only once.
    - The cut is close to the promoter.

3. Review **Table 2A.1** on page 36, and use scissors to cut the plasmid at the restriction site exactly as the restriction enzyme would cut it. Write the sequences of the nucleotides that are left on each end of the plasmid.

4. On the **Human DNA Sequence (RM 3)**, scan the human DNA sequence and determine where the three restriction enzymes—*Bam*HI, *Eco*RI, and *Hin*dIII—would cut the DNA.

5. Determine whether the restriction enzyme you chose in step 2 is a good choice for cutting out the insulin gene from the human DNA by verifying that it meets all the following criteria:
    - It does not cut within the insulin gene
    - It cuts very close to the beginning and end of the gene
    - It will allow the insulin gene to be inserted into the cut plasmid

6. Review **Table 2A.1** and use scissors to cut the human DNA at the restriction site exactly as the restriction enzyme would cut it. Write the sequences of the nucleotides that are left on each end of the insulin gene after it is cut from the human DNA.

7. Use tape to insert the insulin gene into the cut plasmid. Verify that the sticky ends will connect in the correct orientation. (In the lab, a third biological tool, ***DNA ligase***, is used to permanently connect the sticky ends together.) You now have a paper model of a recombinant plasmid that contains an insulin gene. Once the plasmid replicates (copies) itself, the insulin gene is also copied, or cloned!

ACTIVITY QUESTIONS

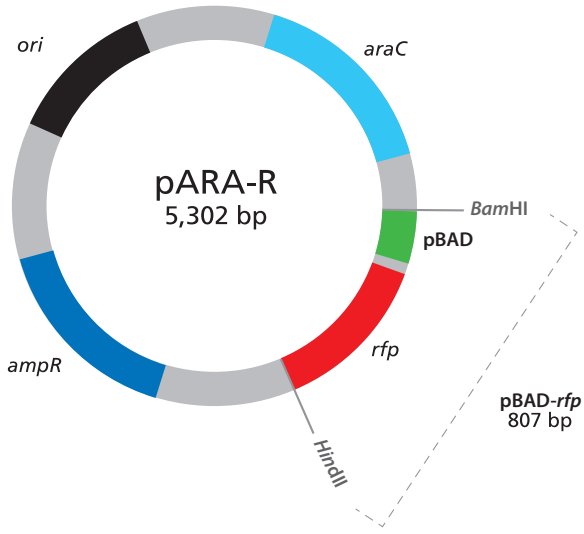
1. Which restriction enzyme did you choose? Why did you choose that one?
2. Where would you insert the insulin gene and why?
3. Which antibiotic would you use to determine if the recombinant DNA was taken in?

LABORATORY 2A: PREPARING TO VERIFY THE *RFP* GENE: DIGESTING THE pARA-R PLASMID

To generate human therapeutic proteins, scientists need to first isolate a fragment of DNA that contains the human gene that codes for the desired protein and then insert that sequence into a plasmid. In this lab, you will do just that. You will ensure that the recombinant plasmid, pARA-R, you have been given is the correct one for making the RFP in bacteria. To do this you will use restriction enzymes to cut the plasmid (see **Figure 2A.5**), which will generate DNA fragments of lengths characteristic of the pARA-R plasmid. This procedure is called a ***restriction digest*** and the lengths of the fragments can be determined by gel electrophoresis (which you may do in Chapter 4A).

The recombinant DNA plasmid pARA-R contains the gene for ampicillin-resistance, the red fluorescent protein (*rfp*) gene, a promoter for initiating transcription, and the *ori* site for the initiation of DNA replication. The pARA-R plasmid also contains a DNA sequence that activates the promoter when the bacteria are grown in the presence of ***arabinose***, a five-carbon sugar that naturally occurs in various plant and bacterial carbohydrates. This sequence is called the arabinose ***activator*** (*araC*). The activator controls the promoter. If arabinose is present in the bacteria, the promoter will bind RNA polymerase, and transcription will occur. In arabinose is not present, the promoter will not bind RNA polymerase, and transcription will not occur.

Figure 2A.5: The pARA-R plasmid



The relevant components on the plasmid are the *rfp* gene, the promoter (pBAD), the ampicillin-resistance gene (*ampR*), and the arabinose activator (*araC*).

In addition to showing the relevant components, **Figure 2A.5** also shows the size of the plasmid (the number in the center, which indicates the number of base pairs [bp]) and the sequences where it can be cut by the restriction enzymes that will be used in the lab. The sites labeled "*Bam*HI" and "*Hin*dIII" represent restriction sites for these two restriction enzymes. (See **Table 2A.1** on page 36.) **Figure 2A.4** (on page 39) shows the insulin gene being inserted in a single restriction enzyme site in the plasmid. In the cloning of the *rfp* gene, two restriction enzymes *(Bam*HI and *Hin*dIII) are used in cutting the plasmid and in isolating the *rfp* gene. Using two different restriction enzymes has advantages: It allows the inserted gene to be oriented in the correct position for transcribing the "sense" strand of DNA (the strand that codes for the protein), and it prevents the plasmid from reforming a circle without the inserted gene. You'll learn more about this if you do Chapter 4A.

STOP AND THINK: Why does using two different enzymes to cut the plasmid prevent the plasmid from reforming a circle without the inserted gene?

BEFORE THE LAB

Respond to the following with your group, and be prepared to share your thoughts with the class.

1. Review **Figure 2A.5**. If pARA-R is digested with *Bam*HI and *Hin*dIII, what fragments are produced? Record the nucleotide sequence of the sticky ends and the length of each fragment (bp), and indicate the genes and other important sequences present on each fragment.

2. To create a plasmid that can produce RFP in bacteria, what components are needed in the plasmid?

3. Bacteria can be killed by an antibiotic unless they carry a plasmid that has the gene for resistance to that antibiotic. (Scientists call these kinds of genes ***selectable markers***; only bacteria that carry this gene will survive exposure to an antibiotic.) If the uptake of DNA by bacteria is inefficient (as discussed in the reading), why is a selectable marker critical in cloning a gene in bacteria?

4. Read through the *Methods* section on pages 45 and 46 and briefly outline the steps, using words and a flowchart.

MATERIALS

Reagents

- A rack with the following:
  - Microfuge tube of 2.5x restriction buffer (2.5xB)
  - Microfuge tube of pARA-R (RP)
  - Microfuge tube of restriction enzymes BamHI and HindIII (RE)
  - Microfuge tube of distilled water (dH_2O)

Equipment and Supplies

- P-20 micropipette
- Tip box of disposable pipette tips
- 2 1.5-mL microfuge tubes
- Permanent marker
- Microcentrifuge (will be shared among all groups)
- 37°C water bath with floating microfuge tube rack (will be shared among all groups)
- Waste container for used tips and microfuge tubes (will be shared among groups)

SAFETY:

- **All appropriate safety precautions and attire required for a science laboratory should be used, including safety goggles. Please refer to your teacher's instructions.**
- **Wash your hands well with soap after completing the lab.**

METHODS

1. Check your rack to make sure that you have all the reagents listed.
2. Use the marker to label two clean microfuge tubes "R+" and "R–." Include your group number and class period on each tube.
3. Review **Table 2A.2**, which summarizes the reagents that you will add in step 4.

Table 2A.2: Addition of reagents to the R+ and R– tubes

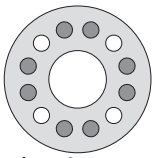
	R+ tube	R– tube
Step 4a: Restriction buffer (2.5xB)	4.0 µL	4.0 µL
Step 4b: pARA-R plasmid (RP)	4.0 µL	4.0 µL
Step 4c: Restriction enzymes (RE)	2.0 µL	
Step 4d: Distilled water (dH_2O)		2.0 µL

LAB TECHNIQUE: In step 4, be sure to use a new micropipette tip for each reagent to avoid contamination.

4. Using a new pipette tip for each reagent, add the following:
   a. 4.0 µL of 2.5xB to the R+ and R– tubes.
   b. 4.0 µL of RP to the R+ and R– tubes.
   c. 2.0 µL of RE to the R+ tube. Add the enzymes directly into the solution at the bottom of the microfuge tube. Gently pump the solution in and out with the pipette to mix the reagents. Cap the tube when done.
   d. 2.0 µL of dH_2O to the R– tube. Gently pump the solution in and out with the pipette to mix the reagents. Cap the tube when done.

STOP AND THINK: In this step, you are asked to set up a tube without the restriction enzymes, *Bam*HI and *Hin*dIII. What is the purpose of this step, and why is it important?

5. Spin the two microfuge tubes (R+ and R–) in the microcentrifuge for four seconds to pool the reagents at the bottom of each tube.



LAB TECHNIQUE: Distribute the tubes evenly in the microcentrifuge so that their weight is balanced.

6. Place both tubes into the 37°C water bath. (You will place your tubes in the floating microfuge tube rack; when the rack is full, your teacher will place it in the water bath.) Incubate for 15 minutes. After the incubation is complete, place both tubes in the freezer at –20°C. You will analyze the contents of the tubes in Laboratory 4A.

STOP AND THINK: Why might the enzymes work best at 37°C? Why should the enzymes then be placed in the freezer? (Hint: The human body temperature is 37°C.)

CHAPTER 2A QUESTIONS

Discuss the following with your partner, and be prepared to share your responses with the class.

1. List in words or indicate in a drawing the important features of a plasmid vector that are required to clone a gene. Explain the purpose of each feature.
2. What role do restriction enzymes have in nature?
3. Using your understanding of evolution, why would bacteria retain a gene that gives them resistance to antibiotics? How is the existence of bacteria with antibiotic resistance affecting medicine today?
4. Bacteria, sea anemones, and humans seem, on the surface, to be very different organisms. How can a gene from humans or a sea anemone be expressed in bacteria to make a product never before made in bacteria?
5. Due to a mishap in the lab, bacteria carrying a plasmid with an ampicillin-resistance gene and bacteria carrying a plasmid with a gene that provides resistance to another antibiotic (kanamycin) were accidentally mixed together. How would you design an experiment allowing you to sort out the two kinds of bacteria? (Hint: Make sure that you do not kill off one of the kinds of bacteria you are trying to sort out!)

CHAPTER 2A GLOSSARY

Activator: A protein that regulates transcription of a gene by binding to a sequence near the promoter, thus enabling RNA polymerase to bind to the promoter and initiate transcription of the gene. The activator protein can also block the binding of RNA polymerase and thereby inhibit transcription of the gene.

Antibiotic: A class of compounds that kill or inhibit the growth of microorganisms.

Antibiotic resistance: The state in which bacteria are no longer sensitive to an antibiotic and will continue to grow and divide in the presence of the antibiotic.

Arabinose: A five-carbon sugar that naturally occurs in various plant and bacterial carbohydrates.

Bacterial conjugation: A process by which two bacterial cells join and transfer genetic material to each other.

Bacterial transformation: The exchange of genetic material between strains of bacteria; a process in which a plasmid is taken up by bacterial cells.

Bacteriophage: A virus that infects a bacterial cell and uses the cell machinery to replicate itself, eventually destroying the bacterial cell.

Base pair: Two complementary nitrogen-containing molecules paired in double-stranded DNA by weak bonds.

Blood clotting factor: A variety of proteins in blood plasma that participate in the clotting process.

Diabetes: A disease that occurs when a person's blood glucose is too high.

Digestion: The cutting of DNA by a restriction enzyme.

DNA cloning: The process of making many exact copies of a particular piece of DNA. (See DNA replication.)

DNA ligase: An enzyme that catalyzes the formation of covalent chemical bonds in the sugar-phosphate backbone, thereby binding together fragments of DNA.

DNA replication: The biological process of making an identical copy of a section of DNA, which occurs each time a new cell is formed in living organisms. The process starts when one double-stranded DNA molecule produces two identical copies. The double helix is unwound, and each strand of the original molecule serves as a template for the production of the complementary strand.

***Escherichia coli* (*E. coli*):** A common bacterium found in the gut of warm-blooded animals. Most strains are harmless, including the strain used in these lab protocols.

Fluorescence: The production of light by a molecule (e.g., red fluorescent protein will release red light when exposed to ultraviolet light).

Growth deficiency: A disease in which a person does not grow properly.

Hemophilia: A disease that occurs when the ability of blood to clot is reduced.

Human growth hormone: A hormone secreted by the pituitary gland that stimulates growth.

Insulin: A hormone produced in the pancreas that controls the amount of glucose in the blood. Insulin is a protein.

Messenger RNA: An RNA molecule transcribed from the DNA of a gene and used as the template for protein synthesis.

Origin of replication (*ori*): A sequence of DNA at which replication of the DNA is initiated.

Promoter: A specific DNA sequence that binds RNA polymerase and initiates transcription of the gene.

Recombinant plasmid: A plasmid built from fragments of DNA from multiple sources.

Red fluorescent protein (RFP): The protein encoded by the *rfp* gene. Mutant fluorescent protein is a molecule that is about 238 amino acids in size. When it is expressed in bacterial cells, the cells appear red or bright pink.

Restriction digest: A technique in which naturally occurring enzymes are used to cleave DNA at specific sequences.

Restriction site (also known as restriction recognition site): A specific DNA sequence that is cut by a restriction enzyme. Many restriction sites are palindromes, sequences that are the same when read forward or backward.

RNA (ribonucleic acid): A single-stranded biomolecule made up of a nitrogenous base, a ribose sugar, and a phosphate; RNA plays a critical role in protein synthesis, transmitting genetic information from DNA to the ribosome where proteins are then made.

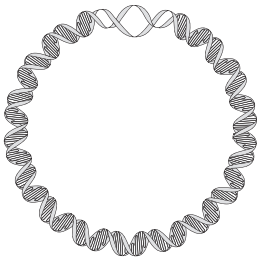
RNA polymerase: A protein that makes messenger RNA from DNA.

Selectable marker: A gene on a plasmid that is introduced into a cell along with a gene of interest that is being cloned. Selectable markers allow scientists to tell if the plasmid has been taken in by the cell because the marker can be seen or detected. A common marker is an antibiotic-resistance gene—only bacteria that have the gene will survive the antibiotic.

Sticky end: End of a DNA molecule cut with certain restriction enzymes. These ends are asymmetrical in that one strand is longer than the other strand and therefore has unpaired bases. The sticky ends of two different pieces of DNA that have been cut with the same restriction enzyme(s) can be joined, as the unpaired bases on their ends are complementary.

Transcription: The process by which information encoded in DNA is transferred to messenger RNA, a single-stranded ribonucleic acid.

Vector: A vehicle for moving DNA sequences from one organism to another.



CHAPTER 4A

MAKING SURE YOU'VE GOT A RECOMBINANT PLASMID

INTRODUCTION

When scientists clone a gene in order to produce a human therapeutic protein, they create a recombinant plasmid that includes the human gene of interest. To do so, they use restriction enzymes to create DNA fragments that contain the plasmid components (Chapter 2A) and then use DNA ligase to join those fragments together. As part of the gene cloning process, scientists have to ***verify*** (confirm) that they have created the recombinant plasmid they need—that is, the one with the gene of interest (which will make the therapeutic human protein) and all the necessary components for that protein to be made. In this chapter, you will continue to work with the tools of genetic engineering as you verify that you have the recombinant plasmid you need in order to produce RFP.

CHAPTER 4A GOALS

By the end of this chapter, you will be able to do the following:

- Describe why it is important to verify products created in the genetic engineering process
- Predict the relative speed of DNA restriction fragments and plasmids through a gel during gel electrophoresis
- Separate and identify DNA restriction fragments and plasmids using gel electrophoresis

WHAT DO YOU ALREADY KNOW?

Discuss the following questions with your partner and write your ideas in your notebook. Be prepared to discuss your responses with the class. Don't worry if you don't know all the answers. Discussing these questions will help you think about what you already know about gel electrophoresis and verification in the lab.

1. Why do DNA restriction fragments and plasmids separate when analyzed by gel electrophoresis?

2. Why is it important to identify and verify a recombinant plasmid?

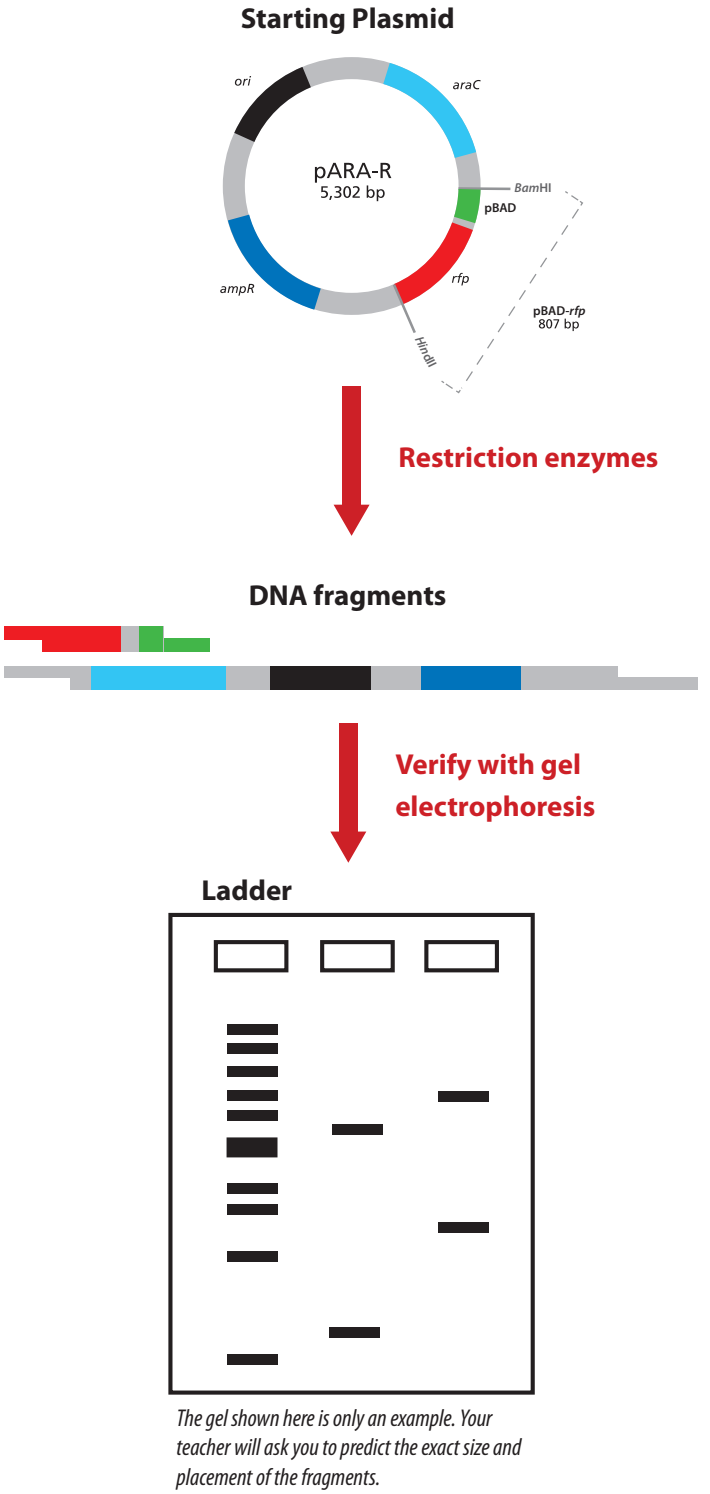
WHY DO YOU NEED TO VERIFY?

Because there are many sources of potential error in any procedure, including the procedures used in cloning a gene, it is important to verify that you have the correct plasmid. In gene cloning, there is also the problem that some procedures are not selective. For example, when a DNA ligase is used to ***ligate***—bind together—DNA fragments, many different combinations result from the ***ligation*** process.

HOW TO VERIFY THE RECOMBINANT PLASMID

Figure 4A.1 shows how to verify the identity of a plasmid. You should compare the results of the restriction digests from Laboratory 2A with a DNA ladder to ensure the plasmid you have been given is the correct plasmid.

Figure 4A.1: Verification method when making a recombinant plasmid

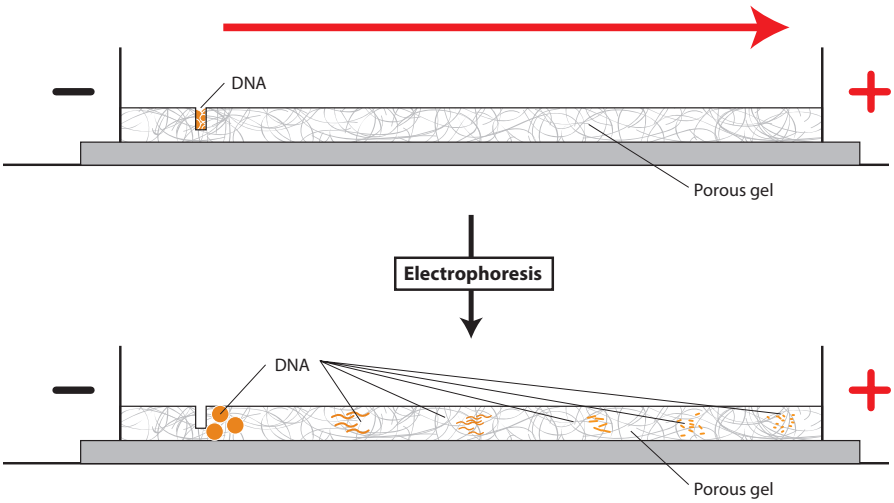


The gel shown here is only an example. Your teacher will ask you to predict the exact size and placement of the fragments.

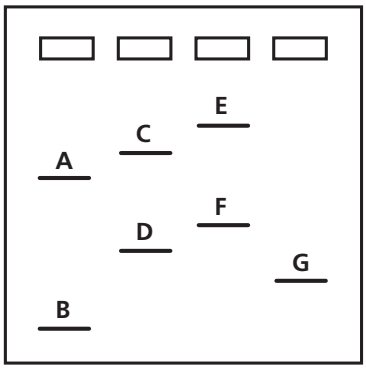
GEL ELECTROPHORESIS

Gel electrophoresis is used extensively in DNA verification and purification. To identify or purify DNA restriction fragments, it is necessary to separate the various-sized DNA molecules. Gel electrophoresis separates biomolecules primarily according to their molecular size, which for DNA is measured by the number of base pairs. The backbone of a DNA molecule, because of its phosphate groups, is negatively charged and therefore will move away from the negative (black) electrode and toward the positive (red) electrode. Because it is easier for small DNA molecules to move through the agarose matrix, they will migrate faster than larger DNA molecules. See **Figure 4A.2**.

Figure 4A.2: Separation of DNA by size using gel electrophoresis



CONSIDER: After different DNA fragments and plasmids have been separated by gel electrophoresis, the gel is stained to show bands that indicate the location of each kind of fragment and plasmid. The drawing of a stained gel below shows a series of bands that have been labeled with letters. The locations of the wells are also shown. What is the order of the fragments, from smallest to largest?



PLASMIDS IN GEL ELECTROPHORESIS

While short, linear pieces of DNA move as expected when run on gel electrophoresis, the movement of plasmids is not as straightforward. This is because a plasmid can exist in different configurations that move at different rates through the gel. There are three plasmid configurations:

- The most common plasmid configuration is ***supercoiled***. You can visualize this configuration by thinking of a twisted rubber band. This twisting or supercoiling results in a very compact molecule, one that will move through the gel very quickly for its size. This configuration is only seen in plasmids that have been replicated in bacteria, because supercoiling of a plasmid requires an enzyme that is found in the bacterial cell. It is the default natural plasmid configuration found in bacteria.

- The second plasmid configuration is a ***nicked circle***. You can visualize this configuration as a large floppy circle. This plasmid has a break in one of the covalent bonds located in its sugar-phosphate backbone along one of the two nucleotide strands. This circular plasmid configuration will not move through the agarose gel as easily as the supercoiled configuration. Although it is the same size, in terms of base pairs, it will be located closer to the well than the supercoiled form.

- The third plasmid configuration is a ***multimer***. You can visualize this configuration by thinking of two or more plasmids that are connected like links in a chain. This configuration is only seen in plasmids that have been replicated in bacteria, because multimers form when plasmids are replicated so fast that they end up linked together. If two plasmids are linked, the multimer will be twice as large as a single plasmid and will migrate very slowly through the gel. In fact, it will move slower than the nicked circle.

The possible plasmid configurations are shown in **Figure 4A.3**.

Figure 4A.3: Plasmid configurations

Supercoiled Nicked circle Multimer

CONSIDER: If you used gel electrophoresis to separate the same plasmid that has all three configurations, which plasmid would move the fastest, and which would move the slowest? Why do the different plasmid configurations move the way they do through the gel? Explain in words or a drawing.

DID YOU KNOW?

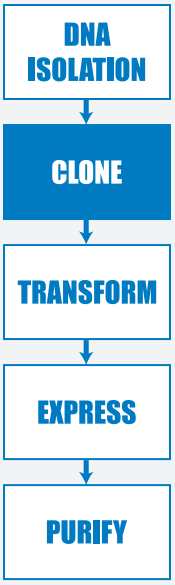
History of Genetic Engineering

Genetic engineering is not a new phenomenon. Throughout history, humans have used selective breeding to produce organisms with desirable traits. The science of agriculture began with the selection of wild grasses and subsequent breeding to form the precursors of modern staples such as wheat, rice, and maize. In ***selective breeding***, two members of the same species are bred together to encourage desirable traits in their offspring. For example, cows that produce large volumes of milk may be bred to pass on that trait to future generations.

The process of inserting or removing DNA from an organism's genome is very new technology, but it holds tremendous promise. With genetic modification, we can increase the nutritional value of staple foods, engineer bacteria to produce therapeutic proteins for use in medicine, and perhaps even disable genes that cause disease.

In organisms produced by traditional selective breeding, the offspring's genome contains genetic information from both parents. In modern genetic engineering, the new organism also contains genetic information from multiple other organisms. Genetic engineering achieves results similar to selective breeding, but more rapidly and with far greater precision.

LABORATORY 4A: VERIFICATION OF THE RECOMBINANT PLASMID USING GEL ELECTROPHORESIS



In this laboratory, you will use gel electrophoresis to examine the products from the restriction digest of the pARA-R plasmid (Laboratory 2A). The sizes of the DNA fragments can be determined by comparing them to a ***DNA ladder***—a mixture of DNA fragments with known sizes. (When the DNA ladder is run on gel electrophoresis and stained, the bands that show the fragments look like the rungs of a ladder.) The DNA ladder is loaded adjacent to other DNA samples to make it easy to compare the bands in the samples with the bands in the ladder. The results from the gel electrophoresis will provide evidence that you are using the pARA-R recombinant plasmid that contains the *rfp* gene. The same procedure would be used to verify that a recombinant plasmid created in the lab contained the gene for a human therapeutic protein.

BEFORE THE LAB

Discuss the following with your group, and be prepared to share your thoughts with the class:

1. The pARA-R plasmid you digested in Laboratory 2A was replicated in a bacterial cell. What configurations—supercoiled, nicked circle, and multimer—might the plasmid have before digestion?

2. You need to predict all the products you might see, including the different plasmid configurations. Review your work in Laboratory 2A. What products might you expect to see in the R– and R+ tubes? Create a table that shows all the possible fragments and plasmids by tube. Include the length (bp size) of each possible fragment or plasmid, and arrange the products found in each microfuge tube by size, from smallest to largest. Include any possible plasmid configurations, and arrange them first by size and next by speed through the gel, from fastest to slowest.

3. Read through the *Methods* section on pages 60 through 62 and briefly outline the steps, using words and a flowchart.

MATERIALS

Reagents

- A rack with the following:
  - Microfuge tube of nondigested pARA-R from Laboratory 2A (R–)
  - Microfuge tube of digested pARA-R from Laboratory 2A (R+)
  - Microfuge tube of loading dye (LD)
  - Microfuge tube of DNA ladder (M)
- 50-mL flask containing 1x SB buffer (shared with another group)

Equipment and Supplies

- P-20 micropipette
- Tip box of disposable pipette tips
- Microcentrifuge (will be shared among all groups)
- Electrophoresis box loaded with 0.8% agarose gel (will be shared among two groups)
- Waste container for used tips and microfuge tubes (will be shared among groups)
- **DNA Ladder Diagram (RM 4)**

SAFETY:

- **All appropriate safety precautions and attire required for a science laboratory should be used, including safety goggles. Please refer to your teacher's instructions.**
- **Wash your hands well with soap after completing the lab.**

METHODS

1. Check your rack to make sure that you have all the reagents listed.
2. Add 2.0 µL of LD to the R– and R+ tubes.

STOP AND THINK: The DNA is not visible as it moves through the gel. The *loading dye* (a set of dyes added to biomolecules for gel electrophoresis) contains the three dyes that you separated in Laboratory 1.2. Why is it useful to use the loading dye in this lab?

3. Spin the R– and R+ tubes in the microcentrifuge for several seconds to pool the reagents at the bottom of each tube.

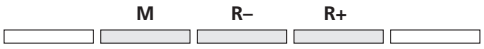
   LAB TECHNIQUE: Distribute the tubes evenly in the microcentrifuge so that their weight is balanced.

4. Make sure that the wells in your gel electrophoresis unit are located near the negative (black) electrode.

5. Fill the box with 1x SB buffer to a level that just covers the entire surface of the gel. If you see any "dimples" over the wells, add more buffer.

   LAB TECHNIQUE: If there are "dimples," add very small amounts of buffer to the electrophoresis box. While the gel needs to be completely under the buffer, you don't want too much buffer in the box, as this will allow the electrical current to run through the buffer and not the gel.

6. Make a drawing in your notebook that shows the location of the wells in the electrophoresis box. The order of the samples in each well is as follows:



7. Using a fresh pipette tip for each sample, dispense 10.0 µL of DNA ladder (M), 10.0 µL of R–, and 10.0 µL of R+ into their designated wells. For each sample, do the following:

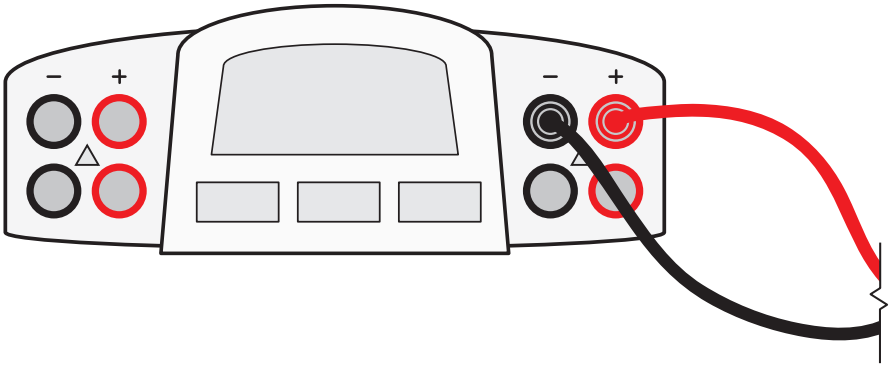
   a. Place your elbow on the table to steady your pipette hand. If needed, also use your other hand to support your pipette hand.
   b. Lower the pipette tip until it is under the buffer but just above the well.

   LAB TECHNIQUE: Do not puncture the gel, or it will become unusable. Gently press the plunger to slowly dispense the sample. To avoid getting air into the well, do not go past the first stop. The sample will sink into the well.

8. When all the samples have been loaded, close the cover tightly over the electrophoresis box.

9. Connect the electrical leads to the power supply. Connect both leads to the same channel, with cathode (–) to cathode (black to black) and anode (+) to anode (red to red). See **Figure 4A.4**.

Figure 4A.4: Leads from electrophoresis box connected to correct channel in power supply



10. Turn on the power supply and set the voltage to 130–135 V.

11. After two or three minutes, check to see if the purple loading dye (bromophenol blue) is moving toward the positive (red) electrode. If it's moving in the other direction—toward the negative (black) electrode—check the electrical leads to see whether they are plugged in to the power supply correctly.

STOP AND THINK:

- **The DNA ladder serves as a standard because it contains a mixture of DNA molecules of known sizes. By running your samples and the DNA ladder side by side in your gel, you can estimate the actual size in base pairs of unknown molecules. The DNA Ladder Diagram (RM 4A) shows 10 DNA bands of known sizes. Using this information, can you predict the positions of DNA bands produced by the possible products found in the R– and R+ tubes by indicating their position on the DNA Ladder Diagram?**
- **The DNA samples and the DNA ladder are not visible on the gel. How might the DNA be made visible once the gel electrophoresis is complete?**

12. Your teacher will explain what to do with your gel. You may not have sufficient time to complete the electrophoresis. The yellow loading dye will need to run just to the end of the gel, about 40–50 minutes. After the gel has finished running, it will need to be stained to show the location of the DNA fragments and plasmids and your teacher will provide you with a photograph of the stained gel to analyze.

CHAPTER 4A QUESTIONS

Analyze your gel photograph and discuss the following questions with your partner. Be prepared to share your answers with the class.

1. Why is it important to verify that you have the correct recombinant plasmid?
2. How did your actual gel results compare to your gel predictions?
3. Do you see any bands that were not expected? What could explain the origin of these unexpected bands?
4. Does the gel show that you are using the correct recombinant plasmid? Describe the evidence you used to make this assessment.
5. In the R– lane, do you see evidence of multiple configurations of plasmids? Explain your answer.
6. In the R+ lane, do you see evidence of complete digestion? Explain your answer.
7. In which lane would you expect to find the *rfp* gene and the *ampR* gene in the gel photograph? Are you able to locate these two genes? Explain your answer.
8. Compare the lanes that have linear fragments with the lanes that have plasmids. Is there a difference in the shape of the bands between these two DNA forms?

CHAPTER 4A GLOSSARY

DNA ladder: A set of known DNA fragments with different sizes in base pairs (bp) or kilo bases (kb). These DNA fragments are separated and visualized as DNA bands on a gel. Together, the separated DNA bands look like a ladder on the gel. DNA ladders are used in gel electrophoresis to determine the size and quantity of DNA fragments.

Ligate: To join together two DNA ends.

Ligation: The reaction that chemically joins two or more fragments of DNA, resulting in a recombinant DNA molecule.

Loading dye: A set of dyes that are added to biomolecules such as DNA for gel electrophoresis. One dye moves farther than the sample, which indicates that it is time to stop running the gel.

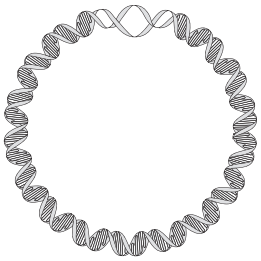
Multimer: A plasmid configuration consisting of multiple plasmids that have interlocked during formation so they are like links in a chain.

Nicked circle: A plasmid configuration that consists of a single plasmid that has a break in one of its two strands. The shape of this plasmid is circular.

Selective breeding: When two members of the same species are bred together to encourage desirable traits in their offspring.

Supercoiled: A plasmid configuration consisting of a single plasmid that has been twisted. The shape of this plasmid is more *compact* (takes up less space) than the circular form.

Verify: Establish that something is true, accurate, or able to be defended.



CHAPTER 5A

GETTING RECOMBINANT PLASMIDS INTO BACTERIA

INTRODUCTION

Once a recombinant plasmid that includes the gene of interest has been created, the next step is to replicate the plasmid and allow the bacteria to produce the protein. Both replication and ***protein expression*** (the way that proteins are synthesized, modified, and regulated in living organisms) can occur only inside a cell. Therefore, your next step in the gene cloning process is to put the recombinant plasmid into *E. coli* bacteria through a process called ***bacterial transformation***, which changes the DNA content of the bacteria. In this chapter, you will carry out the transformation of *E. coli* bacteria using a recombinant plasmid that contains the *rfp* gene. If you were making a human therapeutic protein, the bacteria that you transform would contain the human gene and would be capable of producing the desired human therapeutic protein.

CHAPTER 5A GOALS

By the end of this chapter, you will be able to do the following:

- Describe the role of transformation in the gene cloning process
- Explain the purpose of each control in the transformation experiment
- Explain how the information encoded in a gene is ***expressed*** (the process of converting this information into messenger RNA and then to a protein) as a trait

WHAT DO YOU ALREADY KNOW?

Discuss the following questions with your partner, and write your ideas in your notebook. Be prepared to discuss your responses with the class. Don't worry if you don't know all the answers. Discussing these questions will help you think about what you already know about plasmid uptake and gene expression in bacteria.

1. Do you think that bacterial uptake of a plasmid from the environment is a common event? Why or why not?
2. What are the steps involved in the transcription and ***translation*** (the process by which information encoded in messenger RNA is decoded and transformed into protein) of a gene?
3. What is the relationship among genes, proteins, and traits (or observable characteristics)?
4. What do bacteria and humans have in common that makes it possible for a human gene to be expressed in bacteria?

TRANSFORMING BACTERIA WITH RECOMBINANT PLASMIDS

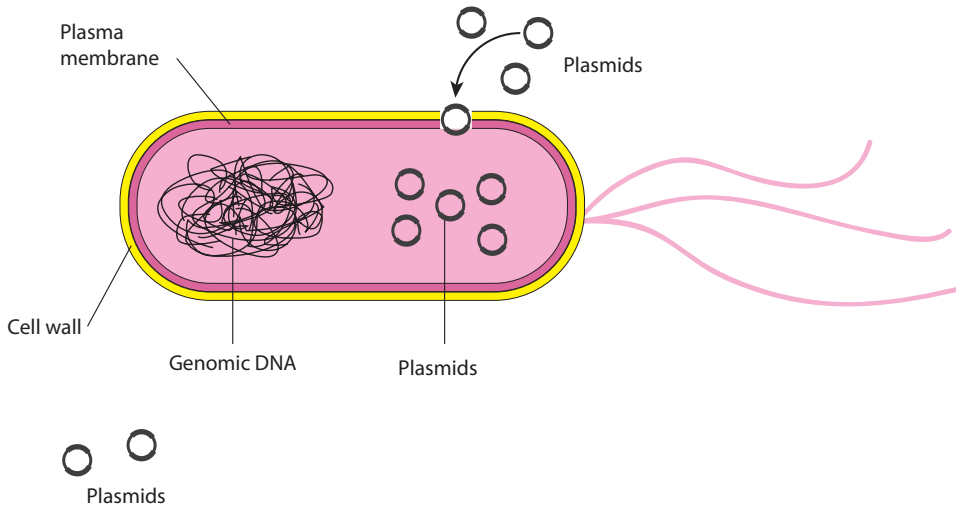
A plasmid is an ideal vector for carrying DNA sequences from one organism to another because it is equipped with (1) a promoter that enables gene transcription, (2) a sequence for the initiation of DNA replication, and (3) an antibiotic-resistance gene. A plasmid can be taken up by bacteria where it replicates, and its genes are expressed using the bacterial cellular machinery. If a gene of interest has been inserted into the vector, the bacteria produces the product encoded by that gene.

CONSIDER: Once a gene has been inserted into a vector, what do you think is required to make the product encoded by the inserted gene?

BACTERIAL TRANSFORMATION

Once a recombinant plasmid is made that contains a gene of interest, such as the insulin gene, the plasmid can enter bacterial cells through the process of transformation. **Figure 5A.1** illustrates transformation.

Figure 5A.1: Bacterial transformation



The uptake of DNA from the environment of a bacterial cell occurs with a very low efficiency in nature. *E. coli* bacteria have complex plasma membranes that separate the external environment from the internal environment of the cell and carefully regulate which substances can enter and exit the cell. In addition, the cell wall is negatively charged and repels negatively charged DNA molecules.

CONSIDER: Why is it important that the membranes of *E. coli* bacteria carefully regulate which substances can enter and exit the cell?

To increase the efficiency of DNA uptake, bacteria are treated in two ways. First, the *E. coli* bacteria are placed in a solution that contains positive calcium ions, which neutralize the negative charge on the cells' outer membranes, enabling DNA molecules to cross the plasma membranes and enter the cell. Next, the bacteria are subjected to a ***heat shock*** (a sudden increase in temperature), which causes the pressure outside the cell to increase. This pressure difference enables the plasmid DNA to enter the bacterial cell from the outside.

Cells treated with calcium and heat are considered ***competent*** (able) to take up DNA more efficiently, but even with this treatment only about 1 in 10,000 bacterial cells takes up a plasmid in its environment. So, how can the bacteria that have taken up the recombinant plasmid be identified? Recall that an important component of these recombinant plasmids is a gene for antibiotic resistance. If you place bacterial cells in the presence of the antibiotic, only those cells that have the recombinant plasmid will grow.

DID YOU KNOW?

A Bacterial Arms Race

Plasmid uptake can have serious consequences in medicine. *Staphylococcus aureus* is a common bacterium that frequently causes infections of the skin and respiratory tract. If the infection is left untreated or spreads to the bloodstream, up to 30% of these infections are fatal.

Some strains of bacteria naturally exchange plasmids, which allows for greater genetic variation in a species that reproduces asexually. One exchange mechanism is ***bacterial conjugation***, in which a plasmid is shared between two bacterial cells that come into contact with one another. The other method is transformation, where bacteria take up DNA directly from their environment. In nature, this is often caused by dead cells releasing their contents into the environment.

Traditionally, doctors have treated staph infections with antibiotics such as vancomycin, which disrupts the formation of bacterial cell walls. However, in recent years, doctors have found that these antibiotics are no longer effective for treating staph infections. New and more aggressive strains of the bacteria are becoming more common, and doctors are unable to treat the infections.

Researchers believe that some *Staphylococcus* bacteria have acquired the *VanA* gene while conjugating with another type of bacteria. The *VanA* gene instills resistance to vancomycin, and certain species naturally carry it within their genome. The resulting vancomycin-resistant *Staphylococcus aureus* bacteria are becoming more commonplace every day and the mortality rate is rising.

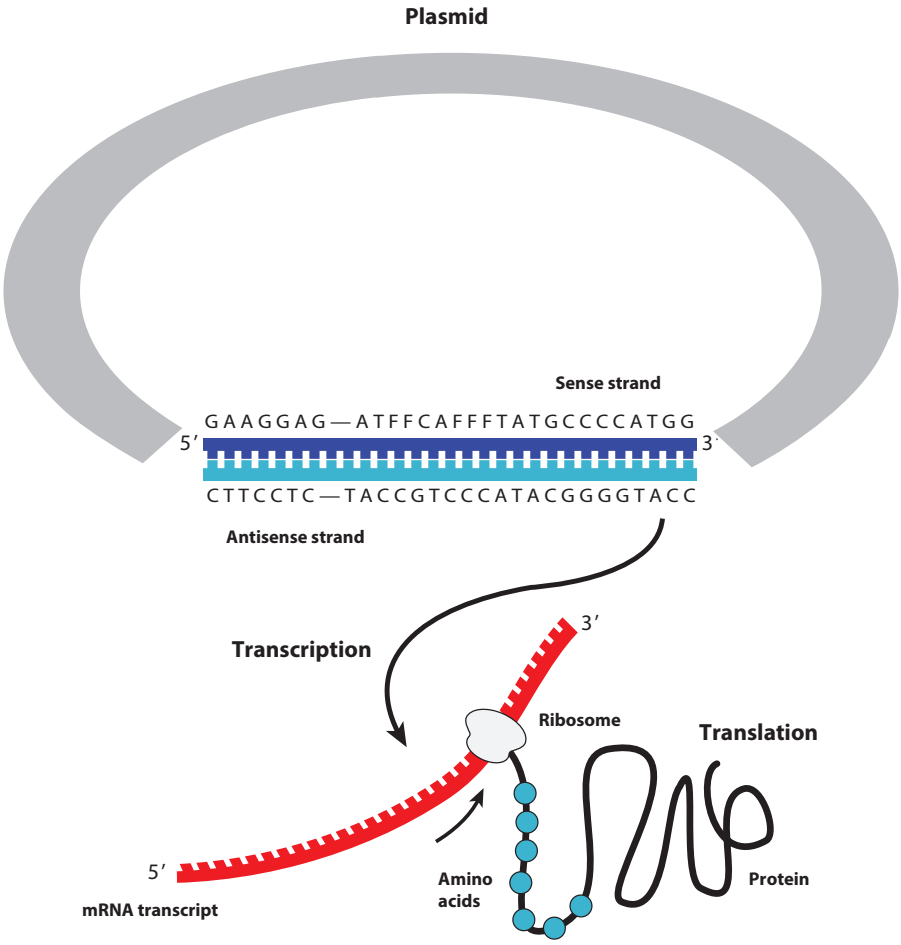
And, the problem of resistant bacteria is not limited to staph. Drug resistant pneumonia and multidrug-resistant tuberculosis have also become increasingly dangerous threats. To stave off the rise of these "super bugs," the biotech industry will need to develop new and innovative treatments.

FROM PLASMID DNA TO PROTEIN

Once a recombinant plasmid has entered the bacterial cell, DNA polymerase initiates replication at the *ori* site, and the plasmid replicates using the bacterial DNA replication enzymes. These multiple copies of plasmids can now produce the protein of interest, such as insulin or human growth hormone, in quantity. In this process, the information encoded in the human DNA is transferred from DNA to protein using the transcription and translation machinery of the cell (see **Figure 5A.2**). The protein then alters the observable traits of the organism.

Genetic engineering is only possible because genes from different organisms can be expressed in bacteria. On Earth, all life is related, and the way that information is encoded in DNA is universal. As you may already know, proteins are made up of smaller subunits called ***amino acids***, and a sequence of three nucleotides in DNA code for a single amino acid. These three-nucleotide sequences are called ***codons***. For example, the codon TTG codes for the amino acid tryptophan, and the codon AAG codes for the amino acid lysine. In many cases, more than one codon can encode the same amino acid. For example, AAA is also a codon for lysine. In addition, there are informational codons, such as the ***start codon*** (ATG) and the ***stop codon*** (TTA), which show where in the DNA sequence the code for the protein begins and ends.

Figure 5A.2: Gene expression from a plasmid in the bacterial cell



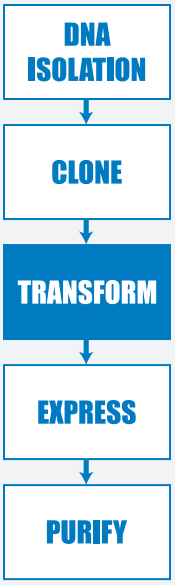
DID YOU KNOW?

Making DNA from RNA

Even though the DNA code is the same in all life forms, the transcription and translation of genes in ***eukaryotes*** and ***prokaryotes*** use different enzymes and structures. (Human cells are eukaryotes, and bacterial cells are prokaryotes.) One important difference between these two kinds of cells is that the genes in eukaryotes contain noncoding sequences called ***introns***. The RNA polymerase transcribes the gene, producing a large precursor messenger RNA containing both introns and ***exons***, which are the coding sequences. The precursor RNA is then ***spliced***, which removes the introns and joins the exons into the mature messenger RNA.

Prokaryotes are unable to carry out the splicing of the introns. To solve this problem, scientists use an enzyme, ***reverse transcriptase*** (which can copy RNA into DNA) to make complementary DNA (cDNA) from the messenger RNA for a particular protein. The cDNA, which has only the exon sequences, is then inserted into the plasmid vector. Cloned human genes used to make human therapeutic proteins are prepared in this way.

LABORATORY 5A: TRANSFORMING BACTERIA WITH THE pARA-R PLASMID

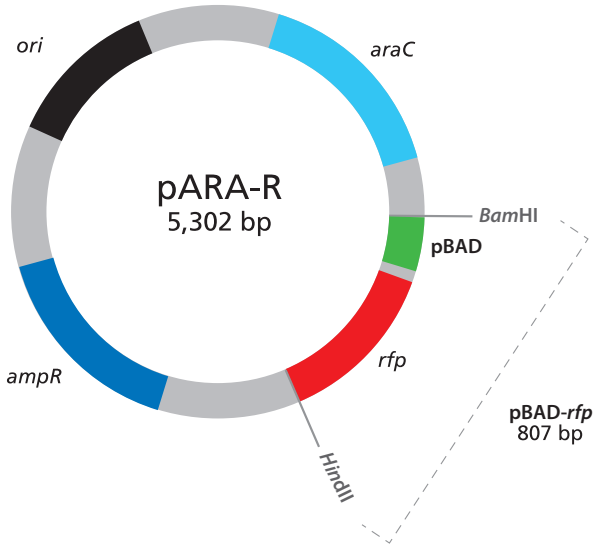


So far in your quest to clone a gene, you have prepared to verify the plasmid by performing a restriction digest (Laboratory 2A), then confirmed the identity of the provided plasmid using gel electrophoresis (Laboratory 4A). In this laboratory you will carry out another step of the gene cloning process: transforming *E. coli* bacteria with the provided plasmid. In the biopharma industry, this process would be used to create the human therapeutic protein. You will divide *E. coli* bacteria that have been pretreated with calcium chloride into two groups: a control group to which no plasmid will be added, and a treatment group to which you will add the pARA-R plasmid. After heat-shocking both groups of cells, you will grow them under several different conditions. All of the bacteria will be grown on three ***agar plates*** (Petri plates containing agar mixed with a ***medium*** or food source named ***Luria Broth*** [LB] that supports bacterial growth). One plate will just contain LB agar, a second will have the antibiotic ampicillin (amp) added to the LB, and a third will include LB, amp, and arabinose sugar (ara) to activate transcription of the protein of interest (RFP).

By examining the growth of bacteria under these conditions, you can verify that your transformation procedure worked, and you can identify the bacteria transformed with the pARA-R plasmid. How will you know if you are successful? The bacteria will have a new and highly visible trait: They will now produce RFP, which makes the cells red or bright pink! If you were making a human therapeutic protein, the bacteria would produce that protein, which would be invisible. However, the products created by the bacteria would be tested to ensure that they contained the desired protein.

The pARA-R plasmid, which you reviewed in Chapter 2A, is shown again in **Figure 5A.3**.

Figure 5A.3: The pARA-R plasmid



The relevant components of this plasmid are the arabinose activator protein gene (*araC*), the promoter (pBAD), the *rfp* gene, and the ampicillin-resistance gene (*ampR*).

Their roles are as follows:

- *araC*: The *araC* gene codes for an activator protein, which turns on the promoter in the presence of arabinose, a simple sugar. (An activator is a protein that regulates transcription of a gene by binding to a sequence near the promoter, thus enabling RNA polymerase to bind to the promoter and initiate transcription of the gene. Activator proteins are used in some recombinant plasmids to control production of the protein of interest.)
- pBAD: pBAD is a promoter.
- *rfp*: The *rfp* gene codes for the expression of red fluorescent protein.
- *ampR*: The *ampR* gene confers resistance to the antibiotic ampicillin.

If arabinose (a sugar) is present, the activator protein from *araC* turns on the promoter. RNA polymerase then binds to the promoter and begins transcription of the *rfp* gene. When the *rfp* gene is expressed, the bacteria turn bright pink. The *ampR* gene is a selectable marker and confers resistance to the antibiotic ampicillin; only bacteria that carry this gene will survive an antibiotic. Only the bacteria containing both the gene for antibiotic resistance, in addition to *araC*, pBAD, and the *rfp* gene, will survive and show the bright pink coloring.

HANDOUTS

- **Bacterial Growth Predictions (RM 5)**

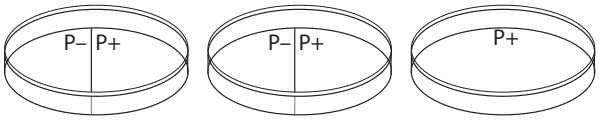
BEFORE THE LAB

Discuss the following with your group, and be prepared to share your thoughts with the class.

1. Ampicillin is an antibiotic that kills bacterial cells by disrupting the formation of cell walls. However, the pARA-R plasmid has the ampicillin-resistance gene, which produces a protein that breaks down ampicillin. What is the purpose of growing bacteria that have been transformed in the presence of ampicillin?

2. What will happen when bacterial cells that contain the pARA-R plasmid are not given arabinose?

3. In the lab, you will add samples of the control group P– and the treatment group P+ to plates that contain various combinations of Luria Broth (LB), ampicillin, and the sugar arabinose. The plates will be arranged as follows:



LB plate LB/amp plate LB/amp/ara plate

   Using the key on **Bacterial Growth Predictions (RM 5)**, show your predictions for the growth you would expect for each combination. Then fill in **Table 1** and **Table 2** in the handout by describing the conclusions that can be drawn if the predicted growth occurs or does not occur.

4. Read through the *Methods* section on pages 77 through 81 and briefly outline the steps, using words and a flowchart.

SAFETY: All appropriate safety precautions and attire required for a science laboratory should be used. Please refer to your teacher's instructions.

SAFETY: Use caution when handling *E. coli* bacteria and use *aseptic technique*.

Aseptic technique is a set of procedures that ensure protection of both the lab worker and the bacterial sample, which is necessary for the experiment to be successful. Specifically:

- **Do not touch anything that has been or will be in contact with *E. coli* bacteria. Students handling equipment that comes into contact with bacteria should wear gloves.**
- **Try to avoid spills or contamination of surfaces with anything that has been in contact with *E. coli* bacteria. Immediately inform your teacher if a spill or contamination occurs.**
- **When you have finished using microfuge tubes, pipette tips, and cell spreaders, place them immediately in the biohazard bag or waste container, as directed by your teacher.**
- **When directed to do so, place your Petri plates in the biohazard bag.**
- **Wash your hands well with soap after completing the lab.**

MATERIALS

Reagents

- A rack with the following:
  - Microfuge tube of pARA-R plasmid (RP)
  - Microfuge tube of Luria Broth (LB)
- Microfuge tube of 100 µL of chilled competent *E. coli* cells (CC)

  NOTE: The CC tube must be kept on ice at all times.

- 3 Petri plates with agar:
  - 1 of LB
  - 1 of LB/amp
  - 1 of LB/amp/ara

Equipment and Supplies

- Styrofoam cup of crushed ice

  NOTE: Fill a cup with some of the crushed ice from the container holding the CC tubes before taking a CC tube. You'll need to keep the CC tube on ice at all times.

- 2 1.5-mL microfuge tubes
- Permanent marker
- Disposable gloves
- P-20 micropipette
- P-200 micropipette
- Tip box of disposable pipette tips
- Pack of cell spreaders (will be shared among groups)
- 42°C water bath with floating microfuge tube rack (will be shared among all groups)
- Timer or clock (will be shared among groups)
- Colored tape (will be shared among groups)
- 37°C incubator (will be shared among all groups)
- Biohazard bag for materials that come into contact with *E. coli* cells (will be shared among groups)
- Waste container (will be shared among groups)

METHODS

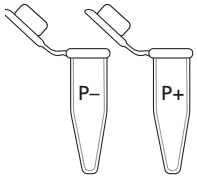
1. Check your rack to make sure that you have the reagents listed.

2. Obtain a CC tube from the ice-filled container, placing it in a Styrofoam cup of ice.

   LAB TECHNIQUE: The competent cells in this lab must be kept cold—be sure to pick up microfuge tubes by the upper rim to avoid warming the cells with your hands.

3. Label two clean microfuge tubes "P–" and "P+."

4. Place the P– and P+ tubes in the Styrofoam cup of ice with the CC tube.



   LAB TECHNIQUE: Bacterial transformation requires sterile techniques. It is essential that these directions be followed precisely.

5. Using the large P-200 micropipette, add the competent cells from the CC tube to the P– and P+ tubes:

   a. Set the P-200 micropipette to 50 µL.
   b. Very carefully, re-suspend the bacterial cells in the CC tube by gently pumping the pipette two times in the solution.
   c. Add 50 µL of CC to each of the empty chilled tubes (P– and P+), holding each tube at its rim to keep it cold, and return each tube quickly to the ice.

   LAB TECHNIQUE: To avoid contamination, be sure to use a new micropipette tip for each addition.

6. Using a new pipette tip, and the P-20 pipette, add RP to the tube labeled "P+":

   a. Set the P-20 micropipette to 10.0 µL.
   b. Hold the chilled P+ tube by the upper rim and add 10.0 µL of RP. Mix the solutions by pumping the pipette two times in the liquids, and return the P+ tube to the ice.

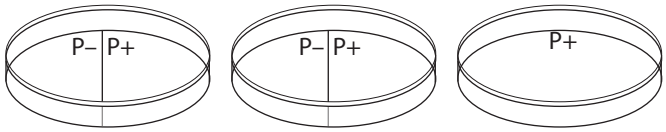
7. Keep the P– and P+ tubes on ice for 15 minutes.

   NOTE: During the 15-minute interval, share and discuss your answers to question 3 in *Before the Lab*.

8. While the cells are on ice, prepare your three agar Petri plates—one plate each of LB, LB/amp, and LB/amp/ara:

   a. Label the bottom of each plate (the part that contains the agar) with your group number and class period. Write small and on the edge of the plate.

b. With the plates closed, draw a line on the bottom of the LB plate and the LB/amp plate that divides each plate in the middle. Label half of each plate "P–" and the other half "P+." Label the LB/amp/ara plate "P+." The plates will be arranged as follows:



LB plate LB/amp plate LB/amp/ara plate

9. Following the 15-minute incubation on ice, carry the P– and P+ tubes (in the cup of ice) to the 42°C water bath. Place the two tubes in the floating microfuge tube rack in the water bath for exactly 45 seconds.

10. After the 45-second heat shock, immediately place the tubes back on ice and leave them there for at least a minute. If the next class will not use the tubes immediately, store them in the refrigerator until ready for use.

11. Using the large P-200 micropipette, add LB to the P– and P+ tubes:

    a. Set the P-200 micropipette to 150 µL.
    b. Add 150 µL of LB to the P– tube. Cap the tube, and gently flick it two or three times to mix.

LAB TECHNIQUE: To avoid contamination, be sure to use a new micropipette tip for each solution.

    c. Add 150 µL of LB to the P+ tube. Cap the tube, and gently flick it two or three times to mix.

12. If time permits, allow the cells in the P– and P+ tubes to incubate at room temperature for 15 minutes.

STOP AND THINK:

- **How is the P+ bacteria culture treated differently from the P– bacteria culture? (A *culture* is an isolated population of cells.) What is the purpose of the P– bacteria culture?**
- **Why do the cells need time to recover after the heat shock?**
- **Why are the cells incubated at 37°C?**
- **You used aseptic technique in this lab. Why is this important?**

13. Add cells from the P– tube to your LB and LB/amp plates:

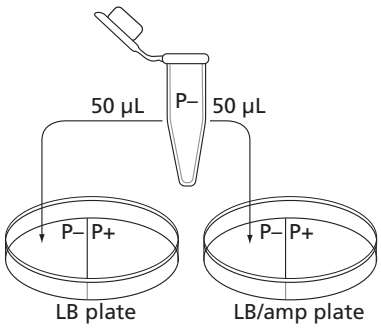
    a. Set the P-200 micropipette to 50 µL.

    LAB TECHNIQUE: To avoid contamination, be sure to use a new micropipette tip for each solution.

    b. Gently pump the pipette two or three times in the P– tube to suspend the cells, and load 50 µL of the P– cells.

    c. Open the lid of the LB plate, like a "clamshell," and add 50 µL of cells from the P– tube to the section marked "P–." Close the lid.

    d. Again, gently pump the pipette two or three times in the P– tube to suspend the cells, and load 50 µL of the P– cells.



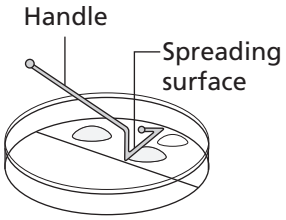
    e. Open the lid of the LB/amp plate, like a clamshell, and add 50 µL of cells from the P– tube to the section marked "P–." Close the lid.

14. Spread the cells from the P– tube on your LB and LB/amp plates:

    a. Open the package of sterile cell spreaders at the end closest to the spreader handles. Remove only one spreader, and close the package to keep the others sterile.

    b. Open the lid of the LB plate, like a clamshell, and spread the cells evenly across the entire P– side of the plate by gently moving the spreader across the agar surface. (Keep the cells on the P– side of the plate.) Close the lid.

    c. Carefully spread the P– cells on the LB/amp plate, using the same spreader and technique.



    LAB TECHNIQUE: Hold the spreader by the handle, and do not allow the bent end to touch any surface, as this will contaminate the spreader. Place the used spreader in the biohazard bag.

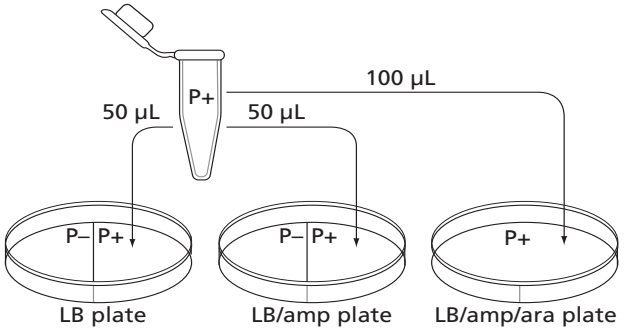
15. Using a new pipette tip, add cells from the P+ tube to your LB, LB/amp, and LB/amp/ara plates:

    a. Make sure that the P-200 micropipette is set to 50 µL.

    LAB TECHNIQUE: To avoid contamination, be sure to use a new micropipette tip for each solution.

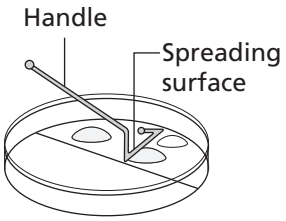
    b. Gently pump the pipette two or three times in the P+ tube to suspend the cells, and load 50 µL of the P+ cells.

c. Open the lid of the LB plate, like a clamshell, and add 50 µL of cells from the P+ tube to the section marked "P+." Close the lid.
d. Again, gently pump the pipette two or three times in the P+ tube to suspend the cells, and load 50 µL of the P+ cells.
e. Open the lid of the LB/amp plate, like a clamshell, and add 50 µL of cells from the P+ tube to the section marked "P+." Close the lid.
f. Set the P-200 micropipette to 100 µL, gently pump the pipette two or three times in the P+ tube, and load 100 µL of the P+ cells.



g. Open the lid of the LB/amp/ara plate, like a clamshell, and add 100 µL of P+ cells to various areas across the surface—not just a single spot. Close the lid.

16. Spread the cells from the P+ tube on your LB, LB/amp, and LB/amp/ara plates:



a. Open the package of sterile cell spreaders at the end closest to the spreader handles. Remove only one spreader, and close the package to keep the others sterile.
b. Open the lid of the LB plate, like a clamshell, and evenly spread the cells on the P+ side of the plate (and only on this side) by gently moving the spreader across the agar surface. Close the lid.
c. Carefully spread the P+ cells on the LB/amp plate using the same spreader and technique.
d. Carefully spread the P+ cells on the LB/amp/ara plate using the same spreader. Then gently rotate the plate beneath the P+ spreader so that the cells can be spread over the entire surface of this plate. Close the lid.

LAB TECHNIQUE: Hold the spreader by the handle and do not allow the bent end to touch any surface, as this will contaminate the spreader. Place the used spreader in the biohazard bag.

17. Allow all three plates to sit right side up for at least five minutes, or until the liquid soaks into the plate.

18. Use the provided tape to tape all three plates together, and label the tape with your group number and class period.

19. Place the plates in the 37°C incubator upside down to prevent condensation from dripping onto the gels.
20. Place all microfuge tubes, pipette tips, and cell spreaders in the biohazard bag.
21. Incubate the plates for 24–36 hours at 37°C. If no incubator is available, the plates may be stored at room temperature for up to 48 hours.
22. Examine the plates. In your notebook, record the amount of growth on each half.
23. Discard the Petri plates in the biohazard bag when directed to do so.

CHAPTER 5A QUESTIONS

1. Look at the results of your transformation. Do your actual results match your predicted results? If not, what differences do you see, and what are some explanations for these differences?
2. How many red colonies were present on your LB/amp/ara plate?
3. Why did the red colonies appear only on the LB/amp/ara plate and not the LB/amp plate?
4. Recombinant plasmids are engineered so that they can replicate in the cell independently of the chromosome replication. Why is it important to have multiple copies of a recombinant plasmid within a cell?
5. How is the information encoded in the *rfp* gene expressed as a trait? Be sure to use what you have previously learned about gene expression and the relationship between DNA, RNA, protein, and traits.
6. Why is it possible for bacteria to make a human protein, such as insulin, or a sea anemone protein, such as RFP?

DID YOU KNOW?

Making the Connection Between Genes and Proteins

How were scientists able to show that a gene codes for a protein? In 1941, George Beadle and Edward Tatum carried out an experiment in which they exposed bread mold to UV irradiation, a procedure known to cause ***mutations*** (changes) in genes. Beadle and Tatum created mutant strains of molds that had lost the ability to synthesize a necessary vitamin. By feeding the precursors of the vitamin one at a time to the mutants, Beadle and Tatum were able to determine that the mutants only lacked a single enzyme catalyzing one reaction.

Beadle and Tatum then investigated whether a single gene caused the loss of the single enzyme by genetic crosses between the mutants and a wild-type strain. After culturing the progeny, they found that half had the same defect as the parent mutant strain and half did not, confirming that a single gene had been mutated. From these results, Beadle and Tatum proposed that genes were responsible for coding the proteins of an organism and that a change in a gene could result in the production of a defective protein, which in turn could affect the traits of that organism. In 1958, Beadle and Tatum received the Nobel Prize for this work.

Understanding the connection between genes and proteins is critical to the advancement of biotechnology. If researchers can gain a greater understanding of which genes impact the proteins involved in a particular disease, they can more effectively work to combat that disease.

CHAPTER 5A GLOSSARY

Agar plate: Petri plate containing agar mixed with a medium or food source named Luria Broth (LB) that supports bacterial growth.

Amino acid: The building block of proteins. Each of the 20 amino acids is an organic substance with two groups attached to it—an amino group (NH_2) and a carboxylic acid group (COOH).

Aseptic technique: A set of procedures and carefully controlled conditions to prevent contamination by pathogens.

Bacterial conjugation: A process by which two bacterial cells join and transfer genetic material to each other.

Codon: A group of three mRNA bases that encode a single amino acid.

Competent: A cell that has the ability to be transformed genetically by taking up DNA from the environment.

Culture: An isolated population of cells that have been grown in a specially prepared nutrient medium.

Eukaryote: An organism that shelters its genes inside a nucleus and has several linear chromosomes.

Exon: The segment of a gene that codes for a protein. Exons are both transcribed and translated.

Expressed: When information encoded in a gene has been converted first into messenger RNA and then to a protein. This process is called expression.

Heat shock: A sudden increase in temperature.

Intron: The segment of a gene that does not code for a protein. Introns are transcribed into mRNA but are removed before the exons (the rest of the gene) are translated into a protein.

Luria Broth: A nutritionally rich medium that supports bacterial growth.

Medium: A solution, such as Luria Broth, that contains substances that support the growth of microorganisms. The medium may be solidified by the addition of agar.

Mutation: Change or damage occurring in a section of DNA that alters the products or processes associated with that section.

Prokaryote: A cell or organism with a single chromosome and no nuclear membrane. Bacteria are prokaryotes.

Protein expression: How proteins are synthesized, modified, and regulated in living organisms.

Reverse transcriptase: An enzyme that catalyzes the formation of DNA from an RNA template in reverse transcription.

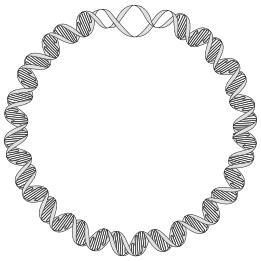
Splice: To modify messenger RNA for translation by removing introns and joining exons.

Start codon: The first codon of mRNA translated by a ribosome, typically AUG or GUG.

Stop codon: A nucleotide triplet within mRNA that signals a termination of translation.

Transformation: A process that places foreign DNA, such as a plasmid, into a cell.

Translation: The process by which information encoded in messenger RNA is decoded and transformed into protein.



CHAPTER 6

GETTING WHAT WE NEED

INTRODUCTION

Genetic engineering is used to produce therapeutic proteins. To provide a treatment for diabetes, for example, a recombinant plasmid is engineered to contain a cloned human insulin gene. Bacteria take up the recombinant plasmid and express the gene, producing insulin. To date, you have carried out all or some of these steps using the cloned *rfp* gene rather than a human gene that would produce a therapeutic protein.

The final step in the process is to obtain the protein. To do this, bacteria are treated with a reagent that ***lyses*** them (breaks open their cell walls), and the protein is separated from the cell contents by a method called ***column chromatography***. (Chromatography is a method for separating similar substances by dissolving them and then flowing the solution over a material that binds the substances to different degrees. Column chromatography uses a column packed with beads coated with the binding material.)

In this chapter, you will complete this final step. You will lyse the bacteria you transformed in Chapter 5 and then use a column that separates proteins based on their solubility in water to obtain RFP made by the cloned *rfp* gene. This same process would be used to isolate a human therapeutic protein.

CHAPTER 6 GOALS

By the end of this chapter, you will be able to do the following:

- Describe the conditions that are favorable to bacterial growth
- Explain how a protein's ***conformation*** (three-dimensional shape) is related to its function
- Explain how ***protein folding*** (the physical process by which a protein folds into its characteristic three-dimensional structure, which is essential to its function) occurs
- Explain how column chromatography separates proteins

WHAT DO YOU ALREADY KNOW?

Discuss the following questions with your partner, and write your ideas in your notebook. Be prepared to discuss your responses with the class. Don't worry if you don't know all the answers. Discussing these questions will help you think about what you already know about bacterial growth and proteins.

1. How do bacteria reproduce?
2. Why are proteins sometimes called workhorse molecules?

3. How might the conformation (shape or folding) of a protein be important for its function? Focus on one of the following protein functions: acting as an enzyme (speeding up reaction rates), transporting molecules, signaling, or forming structures.

4. A ***polypeptide*** is a long linear molecule when it is made, but it immediately folds into a specific three-dimensional conformation, which we call a protein. What properties of the amino acids in a protein control the folding process?

PRODUCING THE PROTEIN OF INTEREST

After transforming the bacteria so that they contain the plasmid with the gene of interest, you need the bacteria to get the bacteria to reproduce and then to express this gene (make the protein that the gene codes for).

BACTERIAL REPRODUCTION

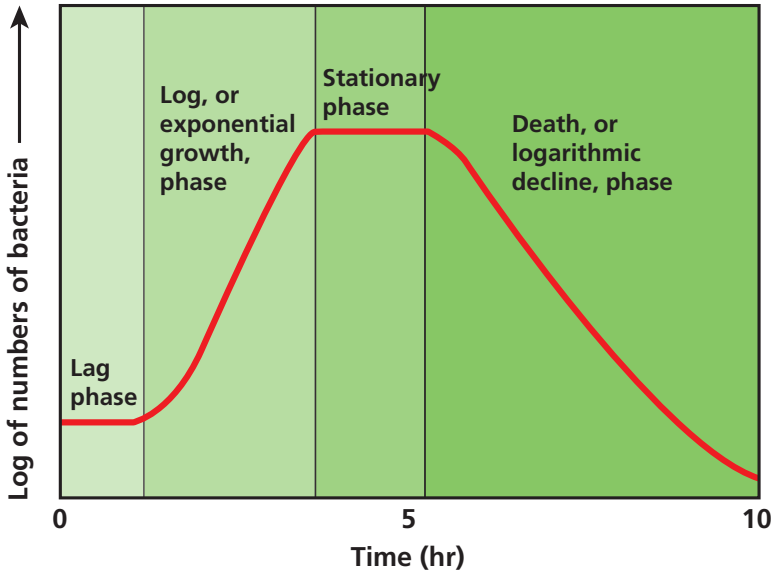
What factors affect bacterial reproduction, which is also called bacterial growth? The last step in Chapter 5 was to place the bacteria that were transformed with the pARA-R plasmid into a ***suspension culture*** in a shaker flask. The cells were suspended in a nutrient broth, and the shaking mixed air into the suspension and prevented the bacteria from settling out of solution, providing conditions favorable to growth.

CONSIDER: Why might the shaker flask be better at supporting bacterial cell growth than a plate?

Under optimal conditions, such as those provided by the shaker flask, the growth of a bacterial population is predictable (see **Figure 6.1**). The growth occurs in four distinct phases:

- In the ***lag phase***, there is a zero growth rate. There are no new cells and no cells dying. The cells adjust to the new conditions, grow larger, and prepare for cell division.
- In the ***log phase***, there is a logarithmic growth rate. The number of new cells is greater than the number of cells dying. The cells undergo asexual cell division and double in number about every 20 minutes (which is the average doubling time for *E. coli*; other bacteria have different doubling times). This phase occurs as long as necessary resources, such as food and oxygen, are unlimited, and there are no unusual factors that cause cell death.
- In the ***stationary phase,*** there is a zero growth rate. The number of new cells equals the number of cells dying. This phase occurs once resources, such as food and oxygen, become limited.
- In the ***death phase***, there is a negative growth rate. The number of new cells is less than the number of cells dying. This phase occurs when resources are depleted and when toxic waste products build up.

Figure 6.1: Change in bacterial growth over time in the shaker flask



CONSIDER: **If the gene of interest is controlled by an operon, such as the arabinose operon, when is the best time to turn on the gene? Keep in mind:**

- **Production of the protein takes energy away from the processes of cell growth and cell division**
- **A greater number of cells will produce more protein**
- **Proteins can degrade over time**

PROTEIN PURIFICATION

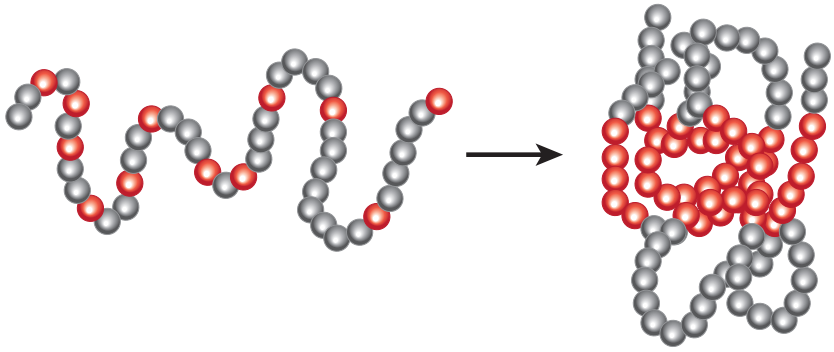
When the transformed bacteria are allowed to grow and multiply numerous times, they can produce enough therapeutic protein to meet the treatment needs of patients. However, the therapeutic protein must be purified, which requires separating it from the other contents of the cell, including other proteins. (A typical bacterium may contain more than 1,000 different kinds of protein.) Column chromatography is one method used to separate the proteins.

What physical characteristics of proteins enable them to be separated by column chromatography? Although all proteins are made up of amino acids, each kind of protein has a specific function and a specific conformation (shape). The conformation relates to function because the outside surface of a protein has specific sites that bind to other molecules. These ***binding sites*** allow proteins to attach to other molecules, which is how proteins can catalyze reactions, transport molecules, provide a signal, and form structures.

When a polypeptide is first synthesized, it is a long flexible chain of amino acids, but it immediately attains its three-dimensional conformation by a process called protein folding (see **Figure 6.2**). Once it has been folded, the polypeptide is called a protein. Protein folding is dependent on the following properties of the amino acids in the protein:

- Formation of weak noncovalent bonds between positively charged and negatively charged side chains of amino acids
- The tendency for ***hydrophobic*** (water-insoluble) amino acids to be buried on the inside of the protein away from water and for ***hydrophilic*** (water-soluble) amino acids to be found on the outside of the protein exposed to water
- Formation of covalent bonds, called ***disulfide bridges***, that occur between sulfur-containing amino acids

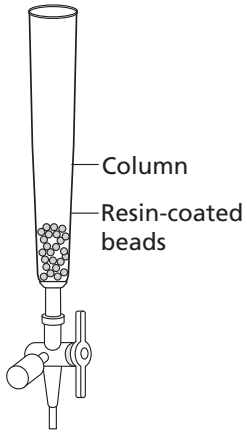
Figure 6.2: Protein folding



Unfolded **Folded**

CONSIDER: If a mutation changes an amino acid, how might this change affect protein folding and protein function?

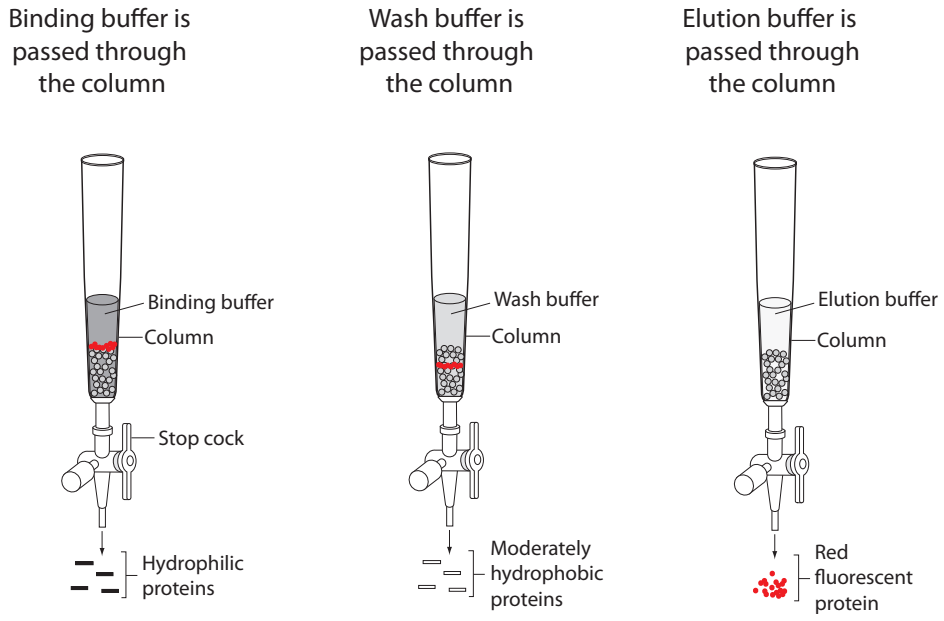
Specific proteins are either hydrophobic or hydrophilic, depending on the relative amount of hydrophobic and hydrophilic amino acids they contain. Hydrophobic proteins and hydrophilic proteins can be separated by column chromatography. In this method, a column is packed with small beads that are coated with a material (called a ***resin***) that attracts hydrophobic amino acids, and the mixture of proteins is dissolved and passed over the beads. For the hydrophobic amino acids to stick to the resin, the proteins must be unfolded to expose the hydrophobic amino acids, which tend to be found on the inside of the protein. Certain salt solutions called ***buffers*** (solutions that resist changes in pH) will unfold proteins to varying degrees.



A series of buffers of different concentrations of salt can be used to separate many proteins from one another. For example, to separate the highly hydrophobic protein

RFP in a column, a binding buffer is used to unfold all the proteins so that hydrophobic proteins stick to the resin and hydrophilic proteins pass through the column. A wash buffer is poured into the column to release moderately hydrophobic proteins from the resin, and then an ***elution*** buffer (used to extract a substance that is bound to another by washing it with a solution) is poured into the column to release RFP from the resin. Both the wash and the elution buffers have a lower salt concentration than the binding buffer and thus cause bound proteins to fold and begin to cover their hydrophobic amino acids. Depending on the extent of folding, the proteins are released from the beads. **Figure 6.3** illustrates this process.

Figure 6.3: Using three buffers to separate red fluorescent protein



CONSIDER: If you were trying to use column chromatography to separate insulin from a mixture of proteins, would you use the same binding, wash, and elution buffers used for RFP, or would you use buffers with different salt concentrations? Explain the reasoning for your answer.

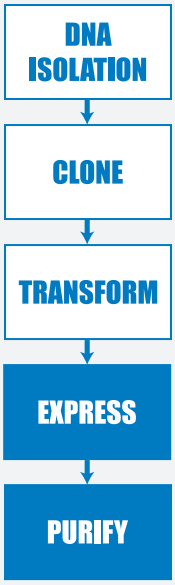
DID YOU KNOW?

Recombinant Proteins

As you have learned, when recombinant proteins are produced for use as human therapeutics, host cells must be grown in large quantities so that enough recombinant protein is produced to meet ***treatment demand*** (the needs of patients). The recombinant protein is isolated, purified, and analyzed for activity and quality before it goes to market.

Producing a protein with the proper order of amino acids isn't always the whole story, however. Sometimes further processing or modification is required to yield an active or fully functioning protein. Many human proteins are ***glycosylated***, meaning that they have a particular pattern of sugar molecules linked to them. If a protein is translated but not correctly glycosylated, it may not function properly. Another modification involves the addition of a phosphate group—a process known as ***phosphorylation***. Phosphorylation of a protein can act as a kind of switch, allowing the protein to become more or less active by uncovering or covering its binding sites. In addition to saccharide (sugar) and phosphate groups, other chemical groups may be added to a protein in order to change its function.

Recombinant proteins for therapeutic use include ***vaccines*** (which cause a body's immune system to make antibodies that will bind to a bacteria, or to make a virus to fight a disease), ***hormones*** (substances that act as chemical messengers in the body), ***monoclonal antibodies*** (proteins that bind to substances in the body and are made by clones of a cell formed in the laboratory), and ***hematopoietic growth factors*** (a group of proteins that promote the growth, differentiation, and activity of blood cells) for the treatment of diseases including cancer, AIDS, allergies, and asthma. The number of recombinant proteins has increased greatly in recent years as the technology used for their production and purification has advanced.



LABORATORY 6: PURIFYING THE FLUORESCENT PROTEIN

In the previous chapter you transformed bacteria and then selected the bacteria that had taken up the plasmid of interest (which included the gene for *rfp*) by placing the cells on plates that contained LB, ampicillin, and arabinose. The bacterial colonies that grew on these plates all contained the *rfp* gene because the *rfp* gene was paired with the gene for ampicillin-resistance. Your class or your teacher then selected one ***colony*** (a group of the same kind of organisms living closely together, usually for mutual benefit), and grew it in a shaker flask to create a large population of identical cells that all contained the recombinant plasmid. Near the end of the log phase of bacterial growth, the cells were given arabinose to turn on the *rfp* genes so that they would make RFP. In the first part of this laboratory, you will use a reagent called lysis buffer to lyse, or break open, the cells. In the second part of this laboratory, you will use column chromatography to isolate, or purify, the RFP. This is the same process that would be used to isolate a human therapeutic protein.

BEFORE THE LAB

Discuss the following with your group, and be prepared to share your thoughts with the class.

1. In column chromatography, how can solutions of different salt concentrations, which will unfold proteins to varying degrees, be used to help purify RFP?

2. Read through the *Methods* sections for Part A (on pages 96 and 97) and for Part B (on pages 98 through 99) and briefly outline the steps, using words and a flowchart.

SAFETY: All appropriate safety precautions and attire required for a science laboratory should be used. Please refer to your teacher's instructions.

SAFETY: Use caution when handling *E. coli* bacteria and use aseptic technique.

Aseptic technique is a set of procedures that ensure protection of both the lab worker and the bacterial sample, which is necessary for the experiment to be successful. Specifically:

- **Do not touch anything that has been or will be in contact with *E. coli* bacteria. Hold microfuge tubes and Petri plates on the outside, hold only the handles of cell spreaders, and do not handle pipette tips at all.**
- **Try to avoid spills or contamination of surfaces with anything that has been in contact with *E. coli* bacteria. Immediately inform your teacher if a spill or contamination occurs.**
- **When you have finished using microfuge tubes, pipette tips, and cell spreaders, place them immediately in the biohazard bag.**
- **When directed to do so, place your Petri plates in the biohazard bag.**
- **Wash your hands well with soap after completing the lab.**

PART A: LYSE CELLS GROWN IN THE SHAKER

MATERIALS

Reagents

- Microfuge tube rack with the following:
  - Microfuge tube of the LB/amp/ara culture of E. coli (EC)
  - Microfuge tube of elution buffer (EB)
  - Microfuge tube of lysis buffer (LyB)
- Additional 1,000 µL (1 mL) of the LB/amp/ara culture of *E. coli* (obtain from your teacher in step 6)

Equipment and Supplies

- Microcentrifuge (will be shared among all groups)
- Liquid waste container
- P-200 micropipette
- Tip box of disposable pipette tips
- Permanent marker
- Waste container (will be shared among groups)
- Biohazard bag for materials that come into contact with *E. coli* cells (will be shared among groups)

METHODS

1. Check your rack to make sure that you have the reagents listed for Part A.
2. Examine the *E. coli* (EC) tube, and record its color in your notebook.
3. Before you can lyse the cells, you will need to separate the cells from the growth medium. To do this, spin the EC tube in the microcentrifuge for five minutes.

LAB TECHNIQUE: Distribute the tubes evenly in the microcentrifuge so that their weight is balanced, making sure that two tubes of the same volume are directly opposite one another.

STOP AND THINK: How can you determine where the RFP is in each separation step?

4. Carefully remove the EC tube from the microcentrifuge to avoid disturbing the solid pellet that contains the bacterial cells.
5. Set the P-1000 micropipette to 800 µL, place a new tip on the micropipette, and carefully remove the ***supernatant*** (the clear liquid found on top of the solid precipitate after a mixture has been centrifuged) from the EC tube without disturbing the cell pellet. (You can dispense the supernatant into the liquid waste container.)
6. Bring the EC tube to your teacher, who will add 1,000 µL (1 mL) of the LB/amp/ara culture of *E. coli* to your EC tube.
7. Repeat steps 3–5 (spin the tube for five minutes and remove the liquid).

STOP AND THINK: What color is the supernatant? The pellet? What are the contents of each?

8. Using the micropipette, carefully remove as much of the liquid as possible without disturbing the pellet, and discard the liquid into the waste container.

LAB TECHNIQUE: Be sure to use a new micropipette tip for each reagent to avoid contamination.

9. Using a new pipette tip and the P-200 pipette, add 150 µL of EB to the cell pellet in the EC tube.

10. Close the cap of the EC tube tightly and vigorously drag the tube across the surface of the microfuge tube rack to resuspend the cells. Examine the EC tube carefully. If there are visible clumps of cells, again drag the tube across the surface of the microfuge tube rack.

11. Using the P-200 pipette, add 150 µL of Lysis Buffer (LyB) to the EC tube. Close the cap of the EC tube tightly and vigorously drag the tube across the surface of the microfuge tube rack two times to mix.

12. Label the EC tube with your group number and class period, and give it to your teacher. Your teacher will incubate the cells at room temperature overnight.

13. Place all microfuge tubes and pipette tips in the biohazard bag.

PART B: SEPARATE THE RED FLUORESCENT PROTEIN WITH COLUMN CHROMATOGRAPHY

MATERIALS

Reagents

- Microfuge tube rack with the microfuge tube of lysed cells (EC, from Part A of this lab)
- Containers with the following:
  - Binding buffer (BB)
  - Wash buffer (WB)
  - Elution buffer (EB)
  - Column equilibration buffer (CEB)

Equipment and Supplies

- 2 1.5-mL microfuge tubes
- Liquid waste container
- P-1,000 micropipette
- Tip box of disposable pipette tips
- Chromatography column
- Microcentrifuge (will be shared among all groups)
- Waste container (will be shared among groups)

METHODS

1. Assign tasks in your group. Have one person make sure that your materials are ready (steps 2–3), another person set up the chromatography column (steps 4–5), and another person spin the lysed cells and remove the supernatant (step 6–7).
2. Check to make sure that you have all the reagents listed.
3. Label two clean microfuge tubes "SUPER" and "RFP."
4. Set up your chromatography column as directed by your teacher, being careful not to dislodge the stopcock attached to the lower portion of the tube.

LAB TECHNIQUE: Do not allow the column to run dry.

5. Prepare the column:
   a. Set the liquid waste collection container under the stopcock.
   b. Carefully open the column by turning the stopcock valve, and allow the liquid to drain into the waste collection container.
   c. Once there is about 1–2 mm of liquid left above the resin bed, close the valve.
   d. Make sure that the liquid is not draining from the column into the waste container.
6. Spin the EC tube in the microcentrifuge for five minutes to create a pellet of the cell debris.

LAB TECHNIQUE: Distribute the tubes evenly in the microcentrifuge so that their weight is balanced.

STOP AND THINK: Three buffers you will use in this lab are the binding buffer (BB), the wash buffer (WB), and the elution buffer (EB). What is the function of each?

7. Examine the tube. You should see a supernatant and a solid pellet.

STOP AND THINK: What color is the supernatant? The pellet? What are the contents of each?

LAB TECHNIQUE: Be sure to use a new micropipette tip for each reagent to avoid contamination.

8. Using the P-1,000 pipette, carefully remove 200 µL of EC supernatant without disturbing the cell debris pellet, and dispense the supernatant into the SUPER tube. If you dislodge the debris pellet, you will have to centrifuge the tube again.
9. Using a new tip, add 200 µL of BB to the SUPER tube. Mix by gently pumping the solution in and out.
10. Using the same tip but changing the volume, add 400 µL of the SUPER tube mixture to the chromatography column. Carefully dispense the solution down the side of the column to avoid disturbing the surface of the resin bed.
11. Open the valve and allow the solution in the column to drain into the waste collection container. Once there is about 1–2 mm of liquid left above the resin bed, close the valve.
12. Examine the column and locate the RFP. Is it spread throughout the resin bed, or does it appear to be restricted to a single band? Record your observations in your notebook.
13. Using a new tip, add 1,000 µL (1 mL) of WB gently down the side of the chromatography column. Try not to disturb the resin.
14. Open the valve and allow the solution in the column to drain into the waste collection container. Close the valve once there is about 1–2 mm of liquid left above the resin bed.
15. Examine the column and locate the RFP. Has the location of the RFP changed in the resin bed?
16. Using a new tip, add 1,000 µL of EB twice (2 mL total), gently, down the side of the chromatography column.
17. Set the RFP tube under the stopcock. Open the valve and allow the part of the ***eluate*** (the solution that washes out) that is red to drain into the RFP tube. Close the valve and cap the tube when done.
18. Set the waste collection container back under the stopcock. Open the valve and allow the rest of the eluate to drain into the waste container. Once there is about 1–2 mm of liquid left above the resin bed, close the valve.
19. Using a new tip, add 1,000 µL of CEB twice (2 mL total) to the chromatography column to prepare it for the next class. Cap the column tightly.
20. Pour the contents of the waste collection container down the sink drain.
21. Compare your RFP tube with RFP tubes from other groups. Is there a difference in intensity of color from sample to sample? Record your observations in your notebook.

CHAPTER 6 QUESTIONS

1. Why is a protein's conformation important for carrying out its function?
2. What properties of the amino acids in a protein relate to protein folding?
3. Does the eluate containing your RFP appear less bright or brighter than it did in the cell lysate following centrifugation? If there is a noticeable difference in the intensity of the red color, what might account for that?
4. What characteristic of RFP is used as the basis for separation by column chromatography?
5. How might the column chromatography procedure be adjusted or modified to increase the purity of the RFP sample?

DID YOU KNOW?

Chimeric Proteins

When you determined which bacteria had taken up the recombinant plasmid that had the *rfp* gene, you were able to see the transformed colony because the bacteria fluoresced red when exposed to light. The ability of a fluorescent protein (FP) to "light up" is a powerful tool that is revolutionizing cell biology and biomedical research. Numerous FPs from different organisms have been isolated and even mutated to provide a toolbox of FP "tags." The FPs can be attached to other molecules to monitor processes that occur inside cells and in the body as a whole.

The function of a protein is directly related to its conformation, which is the result of protein folding. Other important pieces of information that shed light on a protein's role are its distribution, movement, interactions, and association with other proteins. To visualize these aspects of a protein, scientists create a molecule, known as a ***fusion protein*** or a ***chimeric protein***—a chimera is a mythical animal that has parts from different animals—that contains both the protein of interest and an FP fused together. Scientists can measure fluorescence from a single FP, making fusion proteins powerful visualization tools. However, this procedure only works if the FP does not interfere with the protein's function, so tests are run to ensure that a particular protein acts the same with and without the FP tag.

CHAPTER 6 GLOSSARY

Binding site: The area of a biomolecule that coheres to a specific substance or part of a substance.

Buffer: A solution that resists changes in pH, which contains either a weak acid and its salt or a weak base and its salt.

Chimeric protein: A protein created by joining two or more genes that originally coded for separate proteins.

Colony: A group of the same kind of organisms living closely together, usually for mutual benefit. Within a bacterial colony, all organisms descend from a single ancestor and are genetically identical (except for mutations and contamination).

Column chromatography: A method of separating substances, in which the substances are dissolved in a liquid that is allowed to flow through a glass column filled with small beads. The beads are coated with a material that binds the substances to different degrees.

Conformation: The three-dimensional shape or structure of something.

Death phase: The period of bacterial growth in a culture when the bacteria run out of nutrients and die.

Disulfide bridge: A single covalent bond between the sulfur atoms to two amino acids.

Eluate: The solution that washes out (e.g., the solution that drips from a chromatography column).

Elution: The process of extracting a substance that is bound to another by washing it with a solution.

Fusion protein: *See chimeric protein.*

Glycosylation: A process in which a carbohydrate is covalently attached to another molecule.

Hematopoietic growth factor: A group of proteins that promotes the growth, differentiation, and activity of blood cells.

Hormones: Substances that act as chemical messengers in the body.

Hydrophilic: Water-loving; dissolves in water; polar. Some examples are sugar and salt.

Hydrophobic: Water-fearing; does not dissolve in water; non-polar. Some examples are oil, wax, and RFP.

Lag phase: The period of bacterial growth in a culture when the bacteria adapt themselves to growth conditions; individual bacteria are maturing and are not yet able to divide.

Log phase: The period of bacterial growth in a culture when the number of bacterial cells doubles in a fixed period of time (also known as the logarithmic or exponential phase).

Lyse: To break open.

Monoclonal antibody: A type of protein that binds to substances in the body and is made by clones of a cell formed in the laboratory.

Phosphorylation: A process in which an organic compound takes up or combines with phosphoric acid or a phosphorus-containing group.

Polypeptide: A long linear molecule that immediately folds into a specific three-dimensional conformation, which we call a *protein*.

Protein folding: The physical process by which a polypeptide folds into its characteristic three-dimensional structure, which is essential to the protein's function.

Resin: The material used in a chromatography column to coat the beads.

Stationary phase: The period of bacterial growth in a culture when the population stays the same because rates of cell division and cell death are equal. This is often due to a growth-limiting factor, such as the depletion of an essential nutrient.

Supernatant: The clear liquid found on top of a solid precipitate after a mixture has been centrifuged.

Suspension culture: A method of growing cells in a liquid growth medium that is moved around by shaking or stirring. The movement mixes air into the suspension and prevents bacteria from settling out of the solution.

Treatment demand: The needs of patients.

Vaccine: A mixture containing killed or weakened bacteria or viruses that causes a body's immune system to make antibodies that will bind to a bacteria, or to make a virus to fight a disease.